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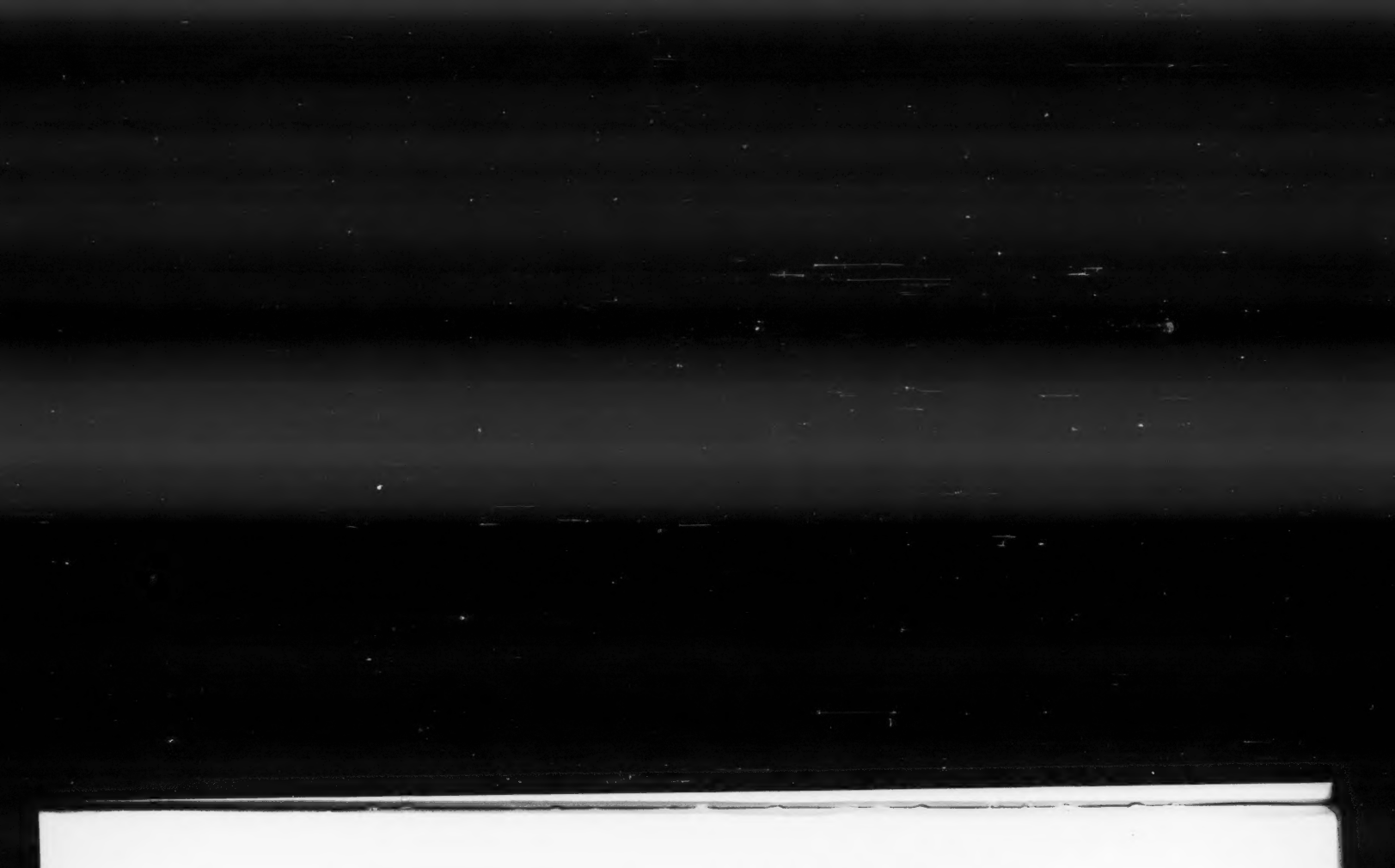

Department of Physiological Chemistry, University of Lund, Lund, Sweden.

On the Conjugation of Bile Acids in the Rat.

Bile Acids and Steroids 14.

By

ARNE NORMAN.

Received 2 February 1954.

Bile acids generally occur conjugated with taurine or glycine in the bile; a recent summary is that of HASLEWOOD and WOOTTON (1950). In order to study the occurrence and conjugation of different bile acids, methods for the quantitative separation of the taurine and glycine conjugates of the common bile acids had to be worked out.

AHRENS and CRAIG (1952) have described the separation by counter-current distribution of some conjugates occurring in ox bile. The reversed phase partition chromatography of HOWARD and MARTIN (1950) has been developed for the separation of free bile acids by BERGSTRÖM and SJÖVALL (1951) and SJÖVALL (1953). A system of more polar phases was used with this technique for the separation of taurocholic, glycocholic and cholic acid by BERGSTRÖM and NORMAN (1953) and the separation of the taurine and glycine conjugates of a number of different acids has been worked out by NORMAN (1953).

This latter technique has been used in this work in which the conjugation of different bile acids in the rat has been investigated with the aid of carboxylabelled cholic, chenodesoxycholic, desoxycholic, lithocholic and cholanic acid. Of these acids only the two first-mentioned have been identified in normal rat bile.

A discussion of earlier work on the conjugation of bile acids in other species will be postponed to a later paper in this series.

Experimental.

The labelled acids are those prepared by BERGSTRÖM, ROTTENBERG and VOLTZ (1952).

Treatment of rats.

The rats used in these experiments were all male albino weighing about 200—250 g.

In order to collect the bile a polythene cannula was inserted in the common bile duct as near the liver as possible. The rats were then placed in small restraining cages and were left with free access to white bread and to a solution of 0.9 per cent sodium chloride. The operative procedure and general routine care of the animals is according to the method described in detail by BERGSTRÖM, SJÖVALL and VOLTZ (1953).

At least 24 hours after the operation, when the rats had recovered and showed a good bile flow, the labelled bile acids were administered intraperitoneally or by gastric intubation under a light ether anesthesia. The bile acids were given as sodium salt dissolved in 2—3 ml of 0.9 per cent sodium chloride. The bile was collected into test tubes with 5 ml ethanol in hourly portions.

Extraction.

The precipitated mucoids were filtered off and the filtrate evaporated to dryness on the water-bath. The residue was dissolved in a small volume of water (15—25 ml) and transferred to a separating funnel. The solution was acidified to pH 1 with hydrochloric acid and extracted three times with 2 volumes of butanol. The combined butanol extracts were then washed with small volumes of water until free of hydrochloric acid. As a consequence of the hydrophilic character of the taurine derivatives a small amount passes over into the water-phase. To recover these the water washings were treated with a second butanol-phase. The combined butanol-phases were then dried with anhydrous Na_2SO_4 and filtered. After evaporation to dryness *in vacuo* the residue was subjected to reversed phase partition chromatography.

The extractions of bile acids from bile were checked at various stages by determining the ^{14}C content. The results are seen below.

1st butanol extract	19,800	c.p.m.
2nd » »	1,500	»
3rd » »	300	»
Acidified aqueous solution after butanol extraction	< 200	»
Combined butanol phases after washing free of HCl	17,770	»
Second butanol phase	3,095	»
Water washings	< 200	»

The first butanol extract of the acidified water-solution of bile acids contains about 95 per cent of the total isotope content. After two further extractions only traces of activity can be found in the water-solution. By washing the combined butanol-phases free from hydrochloric acid a small amount of the conjugated bile acids pass into the water. These

can completely be recovered by passing the washwaters through a second butanol-phase. Separation of the hydrochloric acid from the bile acids by this counter-current distribution is therefore necessary if one desires a complete recovery of the bile acids. Taurine conjugated acids cannot be extracted with ethylacetate or ether from an acidified aqueous solution.

Fractionation.

The residue from the butanol extracts containing bile acids, phospholipids and cholesterol was subjected to reversed phase partition chromatography by the technique described for free bile acids by BERGSTRÖM and SJÖVALL (1951) and SJÖVALL (1953).

It was first possible to separate the taurine conjugated bile acids from the glycine-conjugated and free bile acids using the following solvent system:

Phase system C.

150 ml methanol, 150 ml distilled water, 15 ml iso-octanol, 15 ml chloroform were mixed in a separatory funnel. 4 ml of the lower phase was used per 4.5 g of hydrophobic Supercel.

When the following compounds were chromatographed in this system the peak appeared in the effluent from a column of this size in the volume indicated.

Taurocholic acid	15 ml
Taurodesoxycholic acid	15 ml
Taurolithocholic acid	22 ml
Glycocholic acid	36 ml
Glycodesoxycholic acid	130 ml
Cholic acid	100 ml
Desoxycholic acid	> 250 ml

Fig. 1 illustrates a reference-chromatogram with synthetic taurocholic, glycocholic and cholic acid.

The residue after evaporation of the butanol extract from the bile was dissolved in a few ml of the moving phase. In most cases it was impossible to get everything into solution and a small amount remained undissolved even after slight warming. By placing a 1 cm thick layer of sand on the top of the column the solid particles were filtered off from the substance dissolved in the moving phase. No activity was found to be contained in the solid residue. The size of the columns had to be varied according to the amount of the butanol extract from bile. For 30 mg of total solids from a butanol extract 4.5 g Supercel and 4 ml stationary phase has been used. With greater amounts the Supercel and the stationary phase had to be increased in the same proportion.

The column was eluted with 200 ml moving phase and the effluent was collected in test tubes by using an automatic fraction collector, each fraction containing about 1.5—2 ml. The stationary phase that was then washed out with chloroform contained the cholesterol, the major part of the phospholipids and the less hydrophilic bile acid derivatives viz. dihydroxy, monohydroxy and unsubstituted cholanic

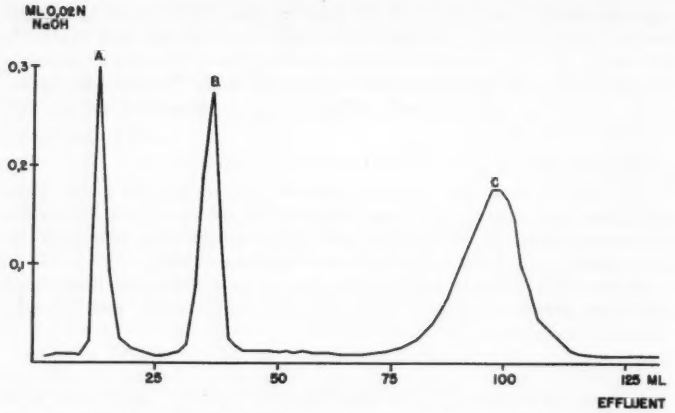

Fig. 1. Chromatography of taurocholic (A), glycocholic (B) and cholic (C) acid. Column: 4.5 g hydrophobic Supercel. Phases: Type C (see page 3).

acids and their keto derivatives and the glycine conjugate of monohydroxy, monoketo or unsubstituted cholanic acid.

The ^{14}C content of the eluate was determined and if this contained any activity it had to be subjected to a new separation with some of the solvent systems suitable to separate the free bile acids.

Phase system A.

180 ml methanol, 120 ml distilled water, 45 chloroform and 5 ml heptane were mixed in a separatory funnel. 4 ml of the lower phase was used per 4.5 g of hydrophobic Supercel.

After titration of the fractions with 0.02 N NaOH in methanol they were each evaporated to dryness in an oven at 90° C. The contents in each test tube was dissolved in 50 % EtOH, quantitatively transferred to a copper planchet and the activity determined. When the tubes contained more than about 0.5 mg of substance a suitable aliquot was used for plating so that the amount plated on each planchet did not exceed this weight. The solid lines in the figures connect the titration values and the open circles connected by a broken line show the c.p.m. of each sample.

For separation of the different tauro compounds which leave the column with the front in solvent system C, the substance on the copper planchets was dissolved in 0.2 N sodium carbonate solution, acidified and extracted with butanol with the above mentioned procedure. The butanol extract is then chromatographed using the following media.

Phase system D.

300 ml water and 100 ml n-butanol were mixed in a separatory funnel. 4 ml of the upper phase was used per 4.5 g of hydrophobic Supercel.

For reference-chromatography with synthetic tauro compounds cf. NORMAN (1953). The order of elution of successive bile acids is taurocholic (20 ml), taurodesoxycholic (32 ml) and taurolithocholic acid (60 ml). The figures in parenthesis state the position of the peak.

Identification of the amino acids conjugated with the bile acids.

The amino acids conjugated with the bile acids were identified according to AHRENS and CRAIG (1952) by paper chromatography in a system consisting of 75 ml of sec-butanol, 15 ml of 88 per cent formic acid.

The fractions containing the bile acids were hydrolysed in a sealed tube with 5 N hydrochloric acid at 100° for 8 hours. The solution was then evaporated to dryness *in vacuo* and the residue stirred in water. The insoluble bile acids were filtered off and an aliquot of the water solution containing the amino-acids was used for paper chromatography.

Results.

A. Conjugation of Bile Acids Normally Present in the Rat Bile.

Cholic acid is the main bile acid present in rat bile (BAYERS and BIGGS 1952, BERGSTRÖM 1952). Chenodesoxycholic acid has been identified as a minor constituent by BERGSTRÖM and SJÖVALL (1954).

2.5—3 mg cholic acid-24-^{14}C were given to rats, which had successfully undergone a bile fistula operation 24 hours previously. This amount corresponds roughly to about ten per cent of the

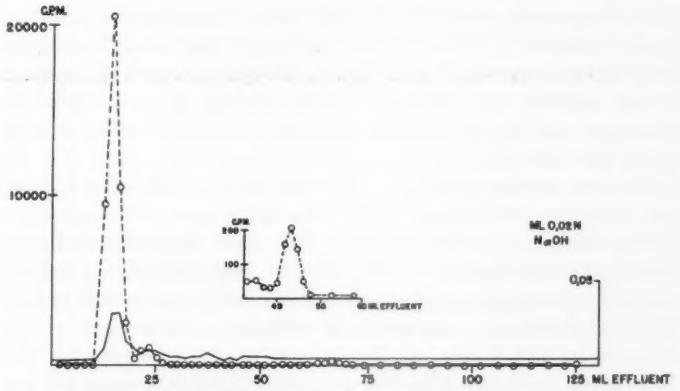

Fig. 2. Chromatography of butanol extract of unhydrolysed bile excreted after intraperitoneal administration of cholic acid-24-^{14}C. Column: 4.5 g hydrophobic Supercel. Phases: Type C (see page 3).

total bile acid present in the rat. As shown by BERGSTRÖM, SJÖVALL and VOLTZ (1953) and BERGSTRÖM, ROTTENBERG and SJÖVALL (1953) the greater part of the isotope content is excreted within the first ten hours and more than 95 per cent after 24 hours. One tenth of the bile excreted during the 24 hours following intraperitoneal administration of labelled cholic acid was analysed by the technique described and the results are shown in fig. 2. Almost all labelled acidic compounds leave the column with the front. Only 1—2 per cent of the activity appears at the position of glycocholic acid, but none at that of free cholic acid. The tauro peak was recromatographed in system D. All activity behaved on the chromatogram as taurocholic acid. The fractions containing the labelled material were combined and recrystallized with 95 mg of unlabelled sodium taurocholate.

The data from the recrystallization appear below:

	Weight of crystals	Crystallized from:	c. p. m. per mg.
1.	100 mg	—	2290
2.	77 mg	90 % EtOH/ether	2325
3.	69 mg	» »	2510
4.	57 mg	» »	2210
5.	50 mg	EtOH/EtOAc	2320
6.	23 mg	» »	2300

The results indicate that taurocholic acid was the only taurocompound present in the bile after i. p. administration of cholic acid-24-^{14}C. The minor band at 40—50 ml effluent was hydrolysed, extracted and again chromatographed in system C. All activity now appeared at the position of free cholic acid. 1—2 per cent of the cholic acid is therefore probably excreted as glycocholic acid.

Several authors (SCHMIDT, HUGHES et al. 1942, BERGSTRÖM and NORMAN 1953) have shown that intestinal microorganisms cause extensive modifications to bile acid molecules and also split taurine conjugates. To see whether these metabolic products are reabsorbed and re-excreted from the liver in a conjugated form, labelled cholic acid was given by stomach tube to rats with a functioning bile fistula so that a part of the labelled acid had first been in contact with the intestinal flora. The excreted bile acids were analysed and the chromatogram showed that all labelled compounds were found conjugated.

The same investigation was made with chenodesoxycholic acid-24-^{14}C. The results were similar, *i. e.* almost all labelled compounds were excreted in the "taurine conjugate band". 1—2 per cent of the activity was present at the position of glycine conjugates, but none at that of free acids.

BERGSTRÖM (1952) and BERGSTRÖM and NORMAN (1953) have shown that the acidic labelled products excreted in the bile after an i. p. injection of cholesterol-4-^{14}C mainly consist of cholic and chenodesoxycholic acid conjugated with taurine. In their experiments the bile fistula was made before the injection of labelled cholesterol and the bile acids analyzed had not been in contact with the intestinal flora. In this experiment a bile fistula was performed seven days after the rat had received an injection of cholesterol-4-^{14}C; hence bile acids which had passed the enterohepatic circulation several times were investigated. Almost all labelled acidic products were again found to be conjugated with taurine.

B. Conjugation of Bile Acids which have not been Identified in Normal Rat Bile.

In a study on the metabolism of bile acids in the rat BERGSTRÖM et al. have investigated the transformation of various less substituted bile acids in the rat liver. After i. p. injection of these bile acids one finds besides some unchanged labelled bile acid a series of labelled metabolic products.

BERGSTRÖM, SJÖVALL and VOLTZ (1953) have shown that after injection of lithocholic-24-^{14}C at least three different acids of more hydrophilic character can be found in the bile besides unchanged lithocholic acid. It was therefore of interest to see to what extent these metabolites were conjugated. Fig. 3 is a chromatogram of the bile acids from a rat given 3.2 mg labelled lithocholic acid. Again practically all the labelled compounds are present as taurine conjugates. The taurolithocholic acid is eluted 5—10 ml after the front containing the taurine conjugates of more hydrophilic acids, causing the second peak. 1 per cent of the activity was found in the eluate. This was chromatographed in system A. All labelled material was eluated at the position of glycolithocholic acid but none at that of free lithocholic acid. The tauro peak was rechromatographed in system D fig. 4. The unchanged lithocholic acid as well as the metabolic products are all excreted as taurine conjugates. The taurine conjugates have been hydro-

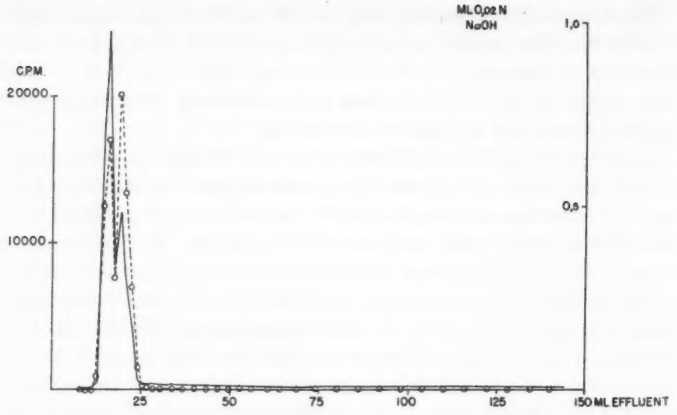

Fig. 3. Chromatography of butanol extract of unhydrolyzed bile excreted after intraperitoneal administration of lithocholic acid-24-^{14}C. Column: 4.5 g hydrophobic Supercel. Phases: Type C (see page 3).

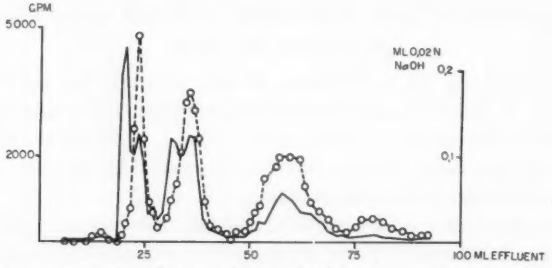

Fig. 4. Chromatography of material from the "taurine conjugate band" (fig. 3). (10—25 ml effluent.) Column: 4.5 g hydrophobic Supercel. Phases: Type D (see page 4).

lysed and the free bile acids chromatographed on system A. The same peaks are found as reported by BERGSTRÖM, SJÖVALL and VOLTZ (1953). When the amount of injected lithocholic acid was increased to 20 mg still no free bile acid could be detected in the bile.

The metabolites and the unchanged form of desoxycholic and cholanic acid were also found to be excreted in the bile as tauroconjugates, with traces of what appeared to be glycineconjugates.

The hydrolysate of the above mentioned "taurine conjugate band" analysed with paper-chromatography showed that taurine was the only ninhydrin positive compound present.

Discussion and Summary.

(24—^{14}C) labelled cholic, chenodesoxycholic, desoxycholic, lithocholic and cholanic acid have been administered intraperitoneally and in some case with stomach tubes to rats and the conjugation of the various bile acids and their metabolites has been investigated.

Bile acids normally present in the bile, *i. e.* cholic and chenodesoxycholic acid are excreted almost totally conjugated with taurine. Only 1—2 per cent of the activity appeared to be glycine-conjugates and no free acids could be detected.

When free cholic or chenodesoxycholic acid was administered *per os* or intraperitoneally these acids or their metabolic products also appeared conjugated in the same way in the bile.

The investigation of the conjugation of the other mentioned bile acids which have not been found in normal rat bile yielded the same results.

It has been shown that hydrolysis of the conjugated bile acids occurs during their passage through the intestine in the rat (Bergström and Norman 1953). Whether this is effected mostly by the digestive enzymes or by the action of the intestinal microorganisms has not been proved.

However, the work presented here has shown that no free bile acids occur in the normal rat after the administration of the different bile acids tested. They are almost totally conjugated with taurine and 1—2 per cent with what seems to be glycine.

This work is part of an investigation supported by "Statens Medicinska Forskningsråd" and by "Knut och Alice Wallenbergs Stiftelse".

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Some Quantitative Analyses of the Sympathomimetic Amine Containing Granules in the Adrenal Medullary Cell.[1]

By

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The sympathomimetic amines in the adrenal medulla are bound to granular cytoplasmic structures which can be isolated from the other cell components as a practically pure fraction (HILLARP and NILSON 1953, HILLARP, LAGERSTEDT and NILSON 1953). As part of the investigations of the structure of these granules and of the way in which adrenaline and noradrenaline are stored in the medullary cell, this work presents some quantitative analyses of the general composition of the granules.

Material and Methods.

The granular fraction was isolated from cow medulla by homogenization according to POTTER-ELVEHJEM in 0.3 M sucrose, followed by differential centrifugation with the technique described in the previous works. To every experiment the suprarenals of 3—6 animals were used. The glands were removed from the body 40—60 minutes after the killing of the animal and immediately cooled by ice.

The homogenization was carried out in such a way that the cell and nuclei free homogenate obtained after the first centrifugation (1×1 min. $+ 1 \times 3$ min. at $800 \times g$) contained approx. 1 g medulla in 8—10 ml. The exact amount of medulla in each homogenate was calculated by determination of the catechol content.

[1] Aided by a grant from Statens Medicinska Forskningsråd.

The homogenates were divided by centrifugation in two fractions: the catechol containing granules and a supernatant which thus contained all the other cytoplasm. The isolated granular fractions contained 77—82 per cent of the total catechol content (adrenaline + noradrenaline) of the various homogenates.

Determination of adrenaline and noradrenaline: The granules in 1.0 ml of nuclei and cell free homogenate were sedimented by centrifugation and suspended in 2.0 ml distilled water which liberates the catechols. The adrenaline and noradrenaline contents were determined directly on 0.4 ml of this suspension and on 0.5 ml of the supernatant colorimetrically according to the method of EULER and HAMBERG (1949 a) with iodine oxidation at pH 4 and 6. In order to stabilize the adreno- and noradrenochrome N acetic acid acetate buffer pH 4 was added, after which the formed protein precipitates were sedimented by centrifugation. l-adrenaline-HCl and l-noradrenaline-HCl were used as standards. Determination of catechols in the adrenal medulla: approx. 0.5 g medulla was ground in a mortar with sand and extracted with 3 N acetic acid. Aliquots of this extract were neutralized to pH 4 and 6 respectively with NaOH and oxidized with iodine.

Determination of lipids: The granules in 20.0 ml homogenate were transferred quantitatively with small amounts of water to approx. 70 ml ethanol-ether (3 : 1, v/v) and boiled for 5 minutes. The alcohol-ether extract was filtered off, and the granules were twice reextracted. Aliquots of the combined extracts were evaporated in N_2 and extracted four times with boiling petroleum ether (b. p. 40—60° C). The lipids in this petroleum ether extract were determined as follows:

Phospholipids: after $MgCl_2$-acetone precipitation by the oxidative micromethod of BLOOR (1929, 1943).

Total fatty acids: oxidative determination according to BLOOR (1928, 1943).

Total cholesterol: by digitonin precipitation after saponification as described by KELSEY (1939).

Free cholesterol: determined in the phospholipid-free acetone extract by precipitation with digitonin according to KELSEY. — In both cases the modified Liebermann-Burchard reagent was used as proposed by SPERRY and BRAND (1943).

Total lipids: the petroleum ether extract was evaporated to dryness in N_2 and the lipids were taken up in chloroform which was centrifuged and evaporated. The residue was weighed.

Determination of wet and dry weight: 4.0 ml homogenate was centrifuged at approx. 7,000 × g during 30 minutes. The granules were washed once with 0.3 M sucrose and again sedimented at 7,000 × g during 60 minutes. The supernatant was removed by suction from the closely packed granules, which were weighed before and after drying to constant weight at 105° C.

Determination of protein-N: The proteins of the granules and of the supernatant were precipitated with 10 per cent trichloroacetic acid and washed twice with 5 per cent TCA. The lipids were removed by three extractions with boiling alcohol-ether (3 : 1). The precipitate

was digested with sulphuric acid — potassium persulphate, after which the protein-N-content was determined according to the Micro-Kjeldahl procedure with titration. The protein content: 6.25 × N.

Results and Discussion.

The distribution of adrenaline and noradrenaline:

In a first series the distribution of adrenaline and noradrenaline within the granules and the supernatant was investigated. The homogenization was here performed in 0.9 M sucrose.

Table I.

The distribution of adrenaline and noradrenaline in eight different homogenates.

Homogenate	Total catechols µg/ml	Granules			The supernatant	
		Per cent of total catechols %	Adrenaline %	Noradrenaline %	Adrenaline %	Noradrenaline %
1	1,220	80	78	22	90	10
2	1,370	79	74	26	89	11
3	1,760	82	78	22	93	7
4	1,110	77	80	20	89	11
5	2,400	78	77	23	85	15
6	1,510	80	75	25	83	17
7	1,600	78	70	30	83	17
8	1,130	79	72	28	82	18
	Average:	79	76 ± 1.2	24 ± 1.2	87 ± 1.4	13 ± 1.4

The results are found in table I. Determinations on eight different homogenates showed that the noradrenaline content of the granules was between 20 and 30 per cent of the total catechol content. This is in good agreement with investigations of EULER and HAMBERG (1949 b) who found 18—31 per cent in cow adrenal medulla. The distribution of the two sympathomimetic amines within the granules and within the remaining cytoplasm was not the same, however. Within the granules adrenaline constituted 76 ± 1.2 per cent and noradrenaline 24 ± 1.2 per cent of the total catechol content, while, on the other hand the corresponding values for the supernatant were 87 ± 1.4 and 13 ± 1.4 per cent respectively. The noradrenaline content was thus significantly lower within the latter fraction.

The adrenaline and noradrenaline that after fractionation of cow medulla is found free in the cytoplasm, is in all probability

released from the granular structures during the isolation procedure (HILLARP, LAGERSTEDT and NILSON 1953). The explanation of the unequal distribution, therefore, seems to be that adrenaline is more readily released from the granules than noradrenaline.

It has not been possible to divide the granules by differential centrifugation in subfractions, within which the distribution of adrenaline-noradrenaline was different from the whole fraction.

Protein, catechol and lipid contents:

By determination of the total catechol content in the medulla, in the isolated granules and in the supernatant combined with determination of the weight of the isolated granules, it is possible to calculate how large a part of the medulla consists of the granules. As can be seen from table II, the wet weight of the granules was 25—29 per cent of the weight of the whole medulla. Assuming that approx. 20 per cent of the medulla consist of connective tissue, blood vessels etc., the catechol containing granules would be one-third of the medullary cell. They are thus an important cell component also from a quantitative point of view. This is evident, too, from the fact that 40—42 per cent of the total cytoplasmic proteins were found in the granules.

The total solids of the granules were on the average 23 per cent of the wet weight, the water content being thus 77 per cent. — The protein content was 32 per cent of the dry weight.

The total amounts of adrenaline and noradrenaline in the granules were high: on the average 19 per cent of the dry weight and 4.3 per cent of the wet weight. As can be further seen from table II, there were approx. 600 μg catechols per mg proteins. This ratio clearly shows the large capacity of the granules for storing sympathomimetic amines.

The determinations of the lipid content in the adrenal medulla are presented in table III. There was nothing here of special interest. On the average there were 19.7 mg phospholipids, 3.2 mg total cholesterol (two-thirds of which were non-esterified), 21.4 mg total fatty acids and 7.5 mg residual fatty acids (calculated as follows: $^2/_3$ weight of the phospholipids + $^2/_3$ weight of the esterified cholesterol were subtracted from the amount of total fatty acids) per g medulla (wet weight).

It has been reported (KUTSCHERA-AICHBERGEN, 1948) that the phospholipid content in the human adrenal medulla is exceptionally high (three times higher than in the suprarenal cortex). As

Table II.

Wet and dry weight, catechol and protein content of eight different isolated granular fractions.

Experiment	Wet weight		Dry weight		Catechol content[1]			Proteins	
	mg	mg/g medulla	mg	Per cent of wet weight %	Per cent of wet weight %	Per cent of dry weight %	µg/mg proteins	Per cent of dry weight %	Per cent of total cytoplasmic proteins %
I	135.0		31.0	23	4.7	20			
II	120.4		28.7	24	4.4	19	650	28	40
III	183.6		38.9	21	3.9	18	540	34	41
IV	172.5		35.6	21	3.8	18	500	37	42
V	183.6		38.5	21	3.7	17	520	34	42
VI	132.4	288	32.3	24	4.6	19	620	30	41
VII	126.9	279	30.8	24	4.5	18	630	30	41
VIII	91.8	249	22.0	24	5.0	21	730	28	40
Average:				23	4.3	19	600	32	41

[1] The values are calculated according to the assumption that all catechol amines in the medulla are bound to the granules (cf. HILLARP, LAGERSTEDT and NILSON, 1953).

can be seen from table III, this was not the case in the cow, however. The phospholipid content of the cortex was on the contrary considerably higher (M = 31.0 mg/g).

In table IV the lipid determinations on the isolated granules are found and compared with determinations on the medulla from which the homogenates were obtained. The total lipids were

Table III.

The lipid content of the adrenal medulla.

Animal No.	Phospholipids mg/g	Cholesterol		Total fatty acids mg/g	Residual fatty acids mg/g	Adrenal cortex Phospholipids mg/g
		Total mg/g	Free mg/g			
1	21.2	2.9	2.1	21.7	7.1	39.0
2	19.8	3.0	2.8	20.6	7.3	32.6
3	18.4	2.8	1.8	20.7	7.7	30.6
4	18.0	3.7	1.9	20.1	6.9	27.4
5	20.7	3.5	2.2	21.4	6.7	31.5
6	20.8	3.5	2.2	22.7	7.9	30.7
7	19.5	3.1	2.2	22.3	8.7	
8—9	19.0					29.2
10—11	20.1					27.2
Average	19.7	3.2	2.2	21.4	7.5	31.0

Table IV.

The lipid content of granular fractions isolated from five different homogenates (20.0 ml).

Experiment	Weight of medulla g	Phospholipids			Total cholesterol		Free cholesterol mg/g medulla	Total lipids Per cent of dry weight %
		mg/g medulla	Per cent of the medullary phospholipids %	Per cent of dry weight %	mg/g medulla	Per cent of the medullary cholesterol %		
VI								
Medulla	0.850	19.1			3.5		2.8	
Granules	2.30 g/20.0 ml	10.6	56	15	1.5	40	1.5	23
VII								
Medulla	1.248	20.5			2.9		2.4	
Granules	2.27 g/20.0 ml	10.4	51	16	1.1	38	1.0	25
VIII								
Medulla	1.083	19.4			2.9		2.1	
Granules	1.84 g/20.0 ml	11.0	57	18	1.0	34	0.9	25
IX								
Medulla	1.519	19.7			2.8		2.5	
Granules	2.82 g/20.0 ml	10.4	53		1.1	40	1.0	
X								
Granules	3.04 g/20.0 ml	10.3			1.6		1.5	
Average			54	16		38		24

approx. one fourth of the dry weight of the granules. The larger part (approx. 67 per cent) were phospholipids (15—18 per cent of the dry weight of the granules) which may be of interest regarding the proposed possibility that the sympathomimetic amines could be bound as phospholipid complexes in the tissues (cf. EULER 1946, 1950, NORLANDER 1951). Somewhat more than half of the medullary phospholipids were found in the granules, while the cholesterol which was practically entirely non-esterified, did not show the same concentration to the granules.

As the catechol containing granules probably are of a mitochondrial nature (HILLARP, HÖKFELT and NILSON 1954) a comparison with mitochondria might be of interest. Values showing good agreement have been obtained, concerning the protein and lipid content of this cell component in various organs. The lipids are 27—30 and the proteins 60—64 per cent of the total solids, and phospholipids are the major component of the lipids (57—79

per cent) (BARNUM and HUSEBY 1948, ADA 1949, SWANSON and ARTOM 1950, KRETCHMER and BARNUM 1951, WATANABE and WILLIAMS 1951). The granules of the medullary cell thus have approx. the same content of total lipids and phospholipids as mitochondria, whereas the protein content is considerably lower.

It is notifiable however, that lipids + proteins + catechols in the medullary granules only represent 75 per cent of the dry weight, whereas the corresponding value for mitochondria is 89—94 per cent. This difference amounting to 14—19 per cent seems to be too large to be caused by different methods of determinations only. There is a possibility, however, that the sympathomimetic amines are not stored in the form of free bases, but as salts of an organic acid.[1] Is that the case the existing discrepancy would be explained.

Summary.

The adrenaline and noradrenaline containing granules in the adrenal medullary cell are an important cell component also from a quantitative point of view. They comprise approx. one-third of the cell weight and contain 40—42 per cent of the total cytoplasmic proteins.

The content of sympathomimetic amines in the granules is very high: on the average 4.3 per cent of the wet weight and 19 per cent of the dry weight. There are on the average 600 μg catechols per mg proteins.

The protein content is on the average 32 per cent of the dry weight. Of the total lipids (approx. 24 per cent of the dry weight) the phospholipids are the major component (approx. 67 per cent) and somewhat more than half the medullary phospholipids are concentrated in the granules. Approx. 38 per cent of the medullary cell cholesterol are found in the granular structures.

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[1] It has been assumed by KENDALL (1932) and UTEVSKI (1936) that the adrenaline in the suprarenal medulla is stored as lactate. Determinations of the amount and localization of the lactic acid in the medullary cell have shown however, that this can not be the case (unpublished observations).

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Inhibitory Effect of Emesis on Water Diuresis in the Dog.

By

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BRUN et al. (1945 a and b) showed that a brief attack of syncope due to circulatory collapse in waterloaded persons caused an antidiuresis lasting for 15 to 90 minutes. The course of the inhibition of the urine flow and the composition of the urine secreted during the period of inhibition gave reason for belief that the oliguria was due to a release of antidiuretic hormone from the neurohypophysis. Recently NOBLE and TAYLOR (1953) showed that persons who fainted during venesection or for other reasons excreted an antidiuretic substance in the urine, but no such substance was found in the urine of persons who became unconscious due to acceleration ("black out").

There thus seems to be reason to believe that a vaso-vagal attack in some way causes a release of hormone from the neurohypophysis. The experiments reported in this paper were therefore undertaken to see if vomiting, which might be looked upon as a mild vaso-vagal attack, had any influence on the water diuresis in the dog.

Methods.

Four mongrel bitches, varying in weight between 8 and 12 kg were used for the experiments. The dogs were perineotomized in order to facilitate catheterization of the bladder. The animals were given 250 to 300 cc of water by stomach tube on the morning of the day of an experiment followed by a second smaller dose of water $3^1/_2$ hours later.

Five hours after the priming dose another 300 cc of water were given and the subsequent water diuresis was followed by collecting the urine continuously from a self-retaining catheter.

Apomorphine hydrochloride given intravenously (in some cases subcutaneously) and copper sulfate ($CuSO_4 \cdot 5H_2O$) given by stomach tube in a one per cent solution were used to induce vomiting. For intravenous injection the apomorphine was dissolved in one cc of distilled water. The solutions of apomorphine were prepared 10 minutes before the injections were made.

In some experiments atropine was given subcutaneously 40 minutes prior to the injection of apomorphine.

On four occasions the arterial blood pressure was recorded during the experiment in either the brachial artery or a branch of the femoral artery under local anaesthesia. Respiratory movements of the thorax were simultaneously recorded with a pneumograph.

In some experiments the amount of chloride in each sample of urine was estimated by the Volhard method.

In one animal (dog no. 2), where the effect of emesis on the water diuresis was repeatedly tested, a stab wound was made between the optic chiasma and the pituitary to a depth of about 0.4 cm. This operation was made under nembutal anaesthesia by the diasphenoid route as described by PICKFORD (1939). The effect of emesis on the water diuresis was then tested again when 14 days had lapsed after the operation.

Results.

A. Influence of Apomorphine Induced Emesis on Water Diuresis and Arterial Blood Pressure.

The intravenous injection of apomorphine in amounts causing vomiting was always followed by an inhibition of the water diuresis lasting from 15 minutes to about one hour. The doses of apomorphine given intravenously to induce vomiting were about 0.01 mg/kg b. w., the time of latency before vomiting occurred was about 2 minutes and no tolerance to the drug seemed to develop — all in accordance with the findings of WANG and BORISON (1950). The vomiting and nausea seen after the injection lasted only for a few minutes, and after that no further signs of nausea were observed. The degree of inhibition of water diuresis seemed to depend more on the intensity of the emesis than on the amounts of apomorphine intravenously injected.

During the inhibition of diuresis the percentage of chloride in the urine was much higher than before and after the inhibition. Fig. 1 A. shows the typical effect of emesis on water diuresis

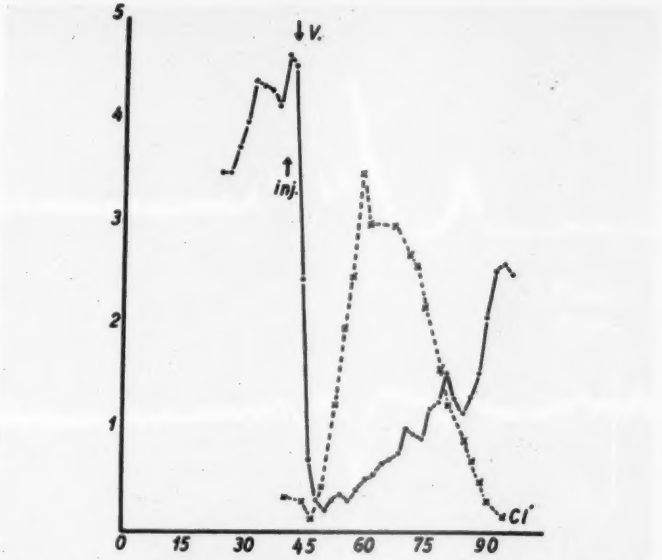

Fig. 1 A. The influence of vomiting, induced by intravenous injection of 0.1 mg of apomorphine hydrochloride, on water diuresis and excretion of chloride in dog no. 3.

inj. = Point of injection.
V = Expulsion of vomitus.
Abscissa: Minutes after the test dose of water given at zero time.
Ordinate: Rate of urine flow in cc/min. mg Cl'/cc of urine (correction in time made for dead space in the catheter and collecting tube of 5.3 cc. No correction made for dead space in the urinary tracts.)

and excretion of chloride after an intravenous injection of 0.1 mg of apomorphine in dog no. 3. Recordings of the blood pressure in the brachial artery and the respiratory movements during the intravenous injection and subsequent emesis are shown in Fig. 1 B. A fall in blood pressure of about 50 mm Hg and slowing of the heart accompanied by great oscillations of blood pressure were seen during retching. The vomitus was expelled near the lowest point of the blood pressure curve, as earlier described by BROOKS and LUCKHARDT (1914) and others.

Injection of 2 mg of atropine subcutaneously 40 minutes prior to the intravenous injection of apomorphine did not seem to influence the inhibition of the urine flow after vomiting or abolish the fall in blood pressure accompanying emesis.

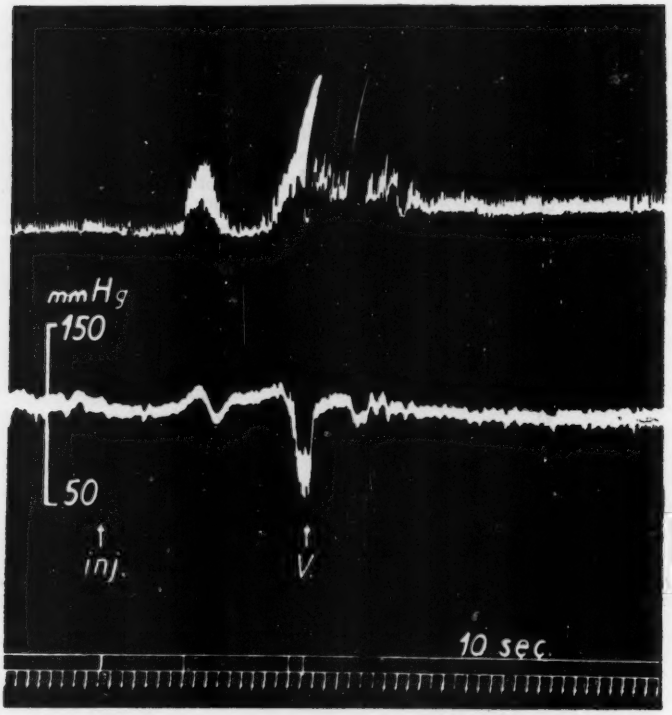

Fig. 1 B. Recordings of the blood pressure in the brachial artery (lower tracing) and respiratory movements during the intravenous injection of 0.1 mg of apomorphine and the subsequent emesis. (Dog no. 3.)
inj. = Point of injection.
V = Expulsion of vomitus.

In a few experiments a much bigger dose of apomorphine (1 mg) was injected subcutaneously during the water diuresis. Such doses of apomorphine resulted in repeated violent retching, vomiting and other signs of nausea lasting for more than half an hour and resulted in complete inhibition of water diuresis for as long as the urine secretion was followed (70 minutes after the injection) (Fig. 2).

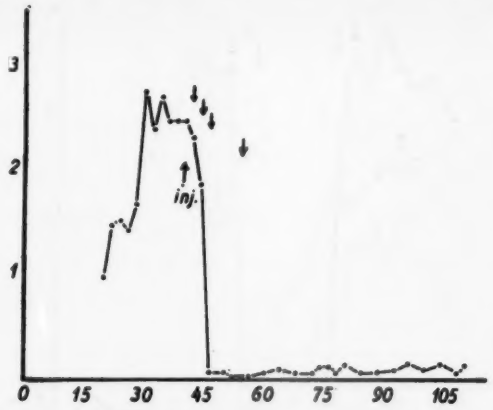

Fig. 2. The effect of vomiting after subcutaneous injection of 1 mg of apomorphine on water diuresis in dog no. 3.
Inj. = Point of injection. (Downwards directed arrows mark the expulsions of vomitus following the injection.)
Abscissa: Minutes after the test dose of water given at zero time.
Ordinate: Rate of urine flow in cc/min.

B. Influence of Copper Sulfate Emesis on Water Diuresis.

In some cases copper sulfate ($CuSO_4 \cdot 5H_2O$) was given by stomach tube 40 minutes after the test dose of water in doses varying from 0.1—0.3 g in a one per cent solution. This resulted in a first emesis after a latent period varying from 10—40 minutes. With doses between 0.2 and 0.3 g the latent period before vomiting occurred was 10—15 minutes, and vomiting was now and then seen during the following 20 minutes. Other signs of nausea lasted still longer and the water diuresis was completely inhibited a few minutes after the first emesis. This inhibition lasted till about 40 minutes after the last emesis, after which time there was a slow and intermittent recovery of the water diuresis (Fig. 3). A smaller dose of copper sulfate gave a prolonged latent period before vomiting occurred, and resulted in fewer expulsions of vomitus during a shorter period and a less pronounced inhibition of water diuresis.

C. The Influence of Emesis on Water Diuresis After Damage to the Supraoptico-Hypophyseal Tract.

After a stab wound between the optic chiasma and the pituitary dog no. 2 showed a definite temporary polyuria, starting within

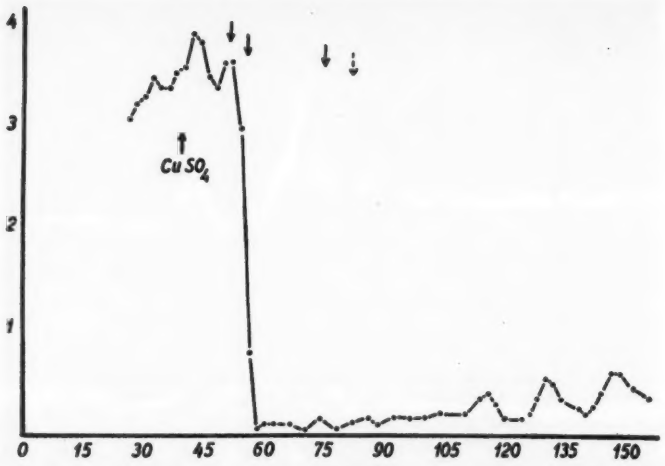

Fig. 3. Inhibition of water diuresis in dog no. 2 after copper sulfate emesis. At the upwards directed arrow 0.25 g of $CuSO_4 \cdot 5H_2O$ was given by stomach tube. Downwards directed arrows mark the expulsions of vomitus.
Abscissa: Minutes after the test dose of water given at zero time.
Ordinate: Rate of urine flow in cc/min.

24 hours after the operation, reaching a peak on the second and third day with a urine secretion of about 1,000 cc of urine in 24 hours and lasting till the 6th day when the urine flow returned to normal and remained there. Thus no permanent diabetes insipidus developed. The response to vomiting after 0.1 mg of apomorphine intravenously was again tested 14 days after the operation. The inhibition of the water diuresis seen in this and subsequent experiments was markedly less than it had been under similar conditions before the operation (Fig. 4).

Discussion.

The course of the inhibition of water diuresis seen after vomiting, the increased concentration of chloride in the urine during the inhibition and the fact that emesis had less inhibitory effect on the water diuresis in one of the animals after damage to the supraoptico-hypophyseal tract indicate that antidiuretic hormone is released from the neurohypophysis during vomiting. From these experiments, however, no conclusions can be drawn as to the nature of the releasing mechanism.

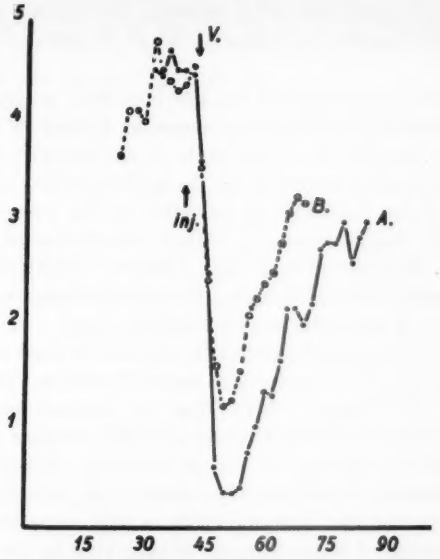

Fig. 4. The effect of emesis after 0.1 mg of apomorphine given intravenously on water diuresis in dog no. 2.

Curve A. = in the intact animal.
Curve B. = 14 days after damage to the supraoptico-hypophyseal tract.
Inj. = Point of injection.
V. = Expulsion of vomitus.
Abscissa: Minutes after the test dose of water given at zero time.
Ordinate: Rate of urine flow in cc/min.

LIM et al. (1937, 1939) reported that electrical stimulation of the central end of the divided vagus nerve caused a liberation of hormone from the neurohypophysis, and electrical stimulation of the central vagi was followed by an ejection of milk in lactating sheep and goats (ANDERSSON 1951). Experimental evidence for the existence of vago-pituitary connections has thus been given, and one possible explanation to the results reported in this paper seems to be that an activation of the medullary center for emesis or afferent impulses set up during the vomiting act should by way of such connections stimulate the neurohypophysis.

Another possible explanation could be that the rapid fall in blood pressure accompanying the act of emesis in some way causes a release of hormone from the neurohypophysis. The rapid fall in hydrostatic pressure seen during vomiting might have the same

effect on osmoreceptors as an increase in osmotic pressure or stimulate the neurohypophysis by way of postulated "volume receptors".

Such a view is supported by the fact that postural fainting causing oliguria in waterloaded persons, as studied by BRUN et al. (1945 a, b), was due to a brief state of circulatory collapse. One possible explanation suggested by the authors in this case was that the effect of the fall in blood pressure on the pressor receptors in some way might involve a reflex stimulation of the neurohypophysis. Further RYDIN and VERNEY (1938) showed that relatively small arterial bleeding in sympathectomized dogs gave an inhibition of water diuresis of pituitary type.

We think it very unlikely that the inhibition observed here was in any of the cases due to emotional stress (RYDIN and VERNEY 1938, O'CONNOR and VERNEY 1945). Thus the animals very soon got used to the experimental conditions and did not show any obvious signs of emotion during the act of vomiting. Vomiting also invariably caused an inhibition of pituitary type, whereas emotional stress (O'CONNOR and VERNEY 1945) is only sometimes followed by an inhibition of water diuresis of that kind in the intact dog.

Summary.

1) Emesis induced by apomorphine and copper sulfate produced inhibition of pituitary type of water diuresis in the dog.

2) 2 mg of atropine given subcutaneously 40 minutes prior to the injection of apomorphine did not abolish this inhibitory effect.

3) Incomplete section of the supraoptico-hypophyseal tract in one of the animals reduced the inhibitory effect of vomiting on the water diuresis.

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Observations on the Electrocardiographic Alterations in the Hibernating Hedgehog.

By

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The most typical feature of the physiology of hibernation is the change of a mammal within certain limits from homoiothermy to poikilothermy (cf. for instance SUOMALAINEN 1953). Thus, during hibernation, the body temperature of the hedgehog may fall to about 1.5°C. During awakening the body temperature rises again in some hours from 1.5—5°C to 33—36°C and the animal returns to homoiothermy.

On account of the low body temperature the metabolism is markedly reduced in a hibernating animal. A manifestation of this phenomenon is the general retardation of the organ functions. For example, it has long been known that the heart rate is greatly reduced during hibernation (cf. for instance HECHT 1915, JOHNSON 1929, ENDRES et al. 1931, FERDMANN and FEINSCHMIDT 1932, BENEDICT and LEE 1938, CHATFIELD and LYMAN 1950). The heart rate in the hibernating hedgehog itself has been reported by BUCHANAN (1911), SUOMALAINEN and SARAJAS (1951), and CHAO and YEH (1951). However, there is no information on the electrocardiographic changes in the hibernating hedgehog. This study is intended to present such data with comparative observations on the electrocardiograms recorded during natural and artificial hibernation.

Artificial hibernation is a state of torpor which can be induced in some hours by injecting insulin into a summer hedgehog, and

then transferring the animal to a cold environment (SUOMALAINEN 1939). This method is based on the observation that a strong hypoglycemia is present in hibernating hedgehogs, and that the islet tissue of the pancreas is at the same time greatly hypertrophied (SUOMALAINEN 1944 and 1952). Furthermore, according to SUOMALAINEN (1953a), this insulin hibernation corresponds in all essential points hitherto investigated to natural hibernation (*e. g.* hypoglycemia, Ca, Mg, and K content of the serum, prolongation of clotting time, etc.). Since the electrocardiogram sensitively reveals alterations and differences in various physiological equilibria, an attempt has been made to confirm the possible identity of natural and artificial hibernation electrocardiographically also.

Animal Material and Methods.

The hedgehog, *Erinaceus europaeus L.*, has been the experimental animal in this investigation. Electrocardiograms were taken with the Siemens Einkoffer electrocardiograph. During hibernation, when the hedgehog lies rolled into a ball with limbs drawn into the protection of the body, the use of the standard limb leads is difficult. Some sort of modification of the limb leads appeared to be the most suitable method. The needle electrodes were pushed about 1.5 cm under the skin, anteriorly to the right and left shoulder blades (regio praescapularis) and into the region of the left gluteal muscles (regio glutea). The leads were placed as follows: Lead I = the right and left r. praescapularis, Lead II = right r. praescapularis — left r. glutea, and Lead III = left r. praescapularis — left r. glutea.

Electrocardiograms were taken from hedgehogs in natural hibernation and from those in artificial hibernation, which had been induced by subcutaneous injection of insulin (1.5—6 I. U.) and placing the animals in a refrigerator, where the temperature was 4—8° C (cf. SUOMALAINEN 1939). In this investigation, artificial hibernation was induced towards the end of August. Electrocardiograms were taken from four hedgehogs in different stages of hibernation, *i. e.* when the animals were entering hibernation in the refrigerator, while the hibernation was at its deepest and, finally, as the animals woke up in room temperature. Electrocardiograms were taken from two animals both during artificial hibernation and after two weeks' natural hibernation. The body temperature (the surface temperature of the abdomen) was measured with a mercury thermometer whilst the ECG was being taken.

During each experimental series the hedgehogs lay on their sides in a little box which was easy to handle. The electrodes remained in the skin of the hedgehog throughout each experimental series. The leads of the electrodes were connected to the coupling holes in the side of the box, to which the coupling wires of the electrocardiograph could be

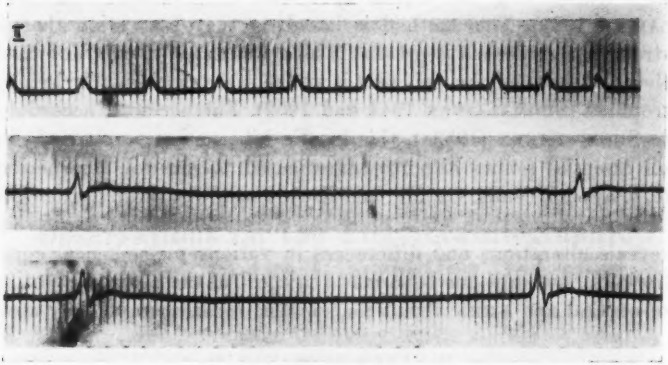

Fig. 1. Lead II in the hedgehog (Table 1, 2, and 3; 770 g) recorded in normal condition (upper curve), during artificial hibernation (middle curve), and during natural hibernation (lower curve).
Time marks = 0.05 sec.

connected when an ECG was taken. The process of recording an ECG took about five minutes. All the time values were measured in Lead II. In all electrocardiograms 1 mV = 15 mm.

Results.

The normal durations of R—R, P, P—R, QRS, and Q—T in the ECG of the hedgehog appear from Table 1. The weight and the body temperature of the animals are also given in the same table. The corresponding heart rate is disclosed in brackets, after the R—R duration. The durations are expressed in 0.01 sec. (see Fig. 1).

The durations of R—R, P, P—R, QRS, and Q—T in the ECG of the hedgehog, recorded during artificial hibernation, are presented in Table 2.

Table 3 reveals the durations of R—R, P, P—R, QRS, and Q—T in the ECG taken from two animals during natural hibernation. This table also shows the corresponding durations during artificial hibernation.

Tables 1 to 3 and Fig. 1 can be summarized as follows: During artificial hibernation, when the body temperature falls from a mean value of 37.2° C to a mean of 7.3° C, the *R—R interval* increases from 32 to 278 or by 768.6 per cent. The *duration of the*

Table 1.

Duration of R—R, P, P—R, QRS, and Q—T in the normal ECG of the hedgehog. The durations are given in 0.01 sec.

Body weight g	Body temperature °C	Duration of					Ratio Q—T/R—R
		R—R	P	P—R	QRS	Q—T	
460	35.2	33 (182)	3.0	5.5	2.3	12.5	0.38
910	35.1	40 (150)	3.0	5.0	2.5	12.0	0.30
510	35.3	23 (265)	2.5	6.0	3.0	12.0	0.52
625	35.1	40 (150)	2.5	6.0	2.5	12.0	0.30
420	35.7	26 (230)	3.0	6.5	3.0	11.0	0.42
770	34.8	40 (150)	2.5	5.5	2.8	13.0	0.33
Mean	35.2	32 (188)	2.8	5.7	2.7	12.1	0.38

Table 2.

Duration of R—R, P, P—R, QRS, and Q—T during artifical hibernation.

Body weight g	Body temperature °C	Duration of					Ratio Q—T/R—R
		R—R	P	P—R	QRS	Q—T	
460	6.6	230 (26)	14.0	34.0	13.0	62.0	0.27
910	7.3	286 (21)	13.5	33.0	14.0	76.0	0.27
510	7.0	280 (21)	12.0	35.0	15.0	65.0	0.24
625	7.3	270 (22)	12.0	36.0	13.0	65.0	0.24
420	6.6	271 (22)	15.0	41.0	16.0	72.0	0.27
770	7.3	330 (18)	12.0	33.0	14.0	102.0	0.31
Mean	7.3	278 (22)	13.1	35.3	14.2	73.7	0.27

Table 3.

Duration of R—R, P, P—R, QRS, and Q—T during natural (N) and artificial (A) hibernation.

Body weight g	Body temperature °C	Duration of					
		R—R	P	P—R	QRS	Q—T	
420	7.7	248 (24)	14.0	35.0	15.0	70.0	N
	6.6	271 (22)	15.0	41.0	16.0	72.0	A
770	6.2	335 (18)	14.0	45.0	15.0	105.0	N
	7.3	330 (18)	12.0	33.0	14.0	102.0	A

P-wave increases from 2.8 to 13.1 or by 367.9 per cent. The *P—R interval* increases from 5.7 to 35.3 or by 517.3 per cent. The *duration of the QRS complex* increases from 2.7 to 14.2 or by 425.5

per cent. The *Q—T interval* increases from 12.1 to 73.7 or by 507.4 per cent. The *ratio Q—T/R—R* decreases from 0.38 to 0.27. Electrocardiograms recorded during artificial and natural hibernation are identical (Fig. 1).

Electrocardiograms recorded during various stages of artificial hibernation show that the changes in body temperature and the electrocardiographic alterations are correlated in a certain way. Figure 3 shows the durations of various waves and intervals in four hedgehogs, plotted against body temperature, and in Fig. 2 serial tracings, taken from a hedgehog in different stages of artificial hibernation, are presented. Of these figures the following notes can be made.

The normal sequence of P—QRS—T is present in every tracing obtained from animals in different stages of artificial hibernation. Apart from the respiratory arrhythmia, no other rhythm changes could be observed. Hence these results constitute evidence that the impulse formation in the heart remains nomotopic during hibernation. With the exception of an occasional inversion of the P wave (Fig. 2: 4), the amplitudes of various deflections appeared to be tolerably constant. It is of significance, for instance, that the serial records reveal neither RS—T segment displacements nor alterations in the voltage of the T wave. Nor did the QRS voltage show any noteworthy changes. As a rule, however, the P wave becomes bifid at low body temperature, and concomitantly the QRS deflections show a slight slurring and/or notching. The aforementioned electrocardiographic changes take place in conjunction with the following duration changes. The decrease in the body temperature is associated with progressive prolongation of the R—R interval to about 15° C and vice versa. Below this temperature level the R—R duration appears to be more or less independent of the body temperature, reflecting the slow change of the heart rate. On the other hand, the lengthening of P, P—R, QRS, and Q—T in various stages of artificial hibernation follows fairly exactly the decrease in the body temperature and vice versa. The lower the body temperature, the greater is the change per 1°C. It can be established, however, that in emergence from hibernation these durations have a tendency to be smaller than at a corresponding temperature in animals entering hibernation. During the process of awakening the R—R interval also appears to be shorter, not attaining its normal level before the body temperature is approximately normal. Finally, the

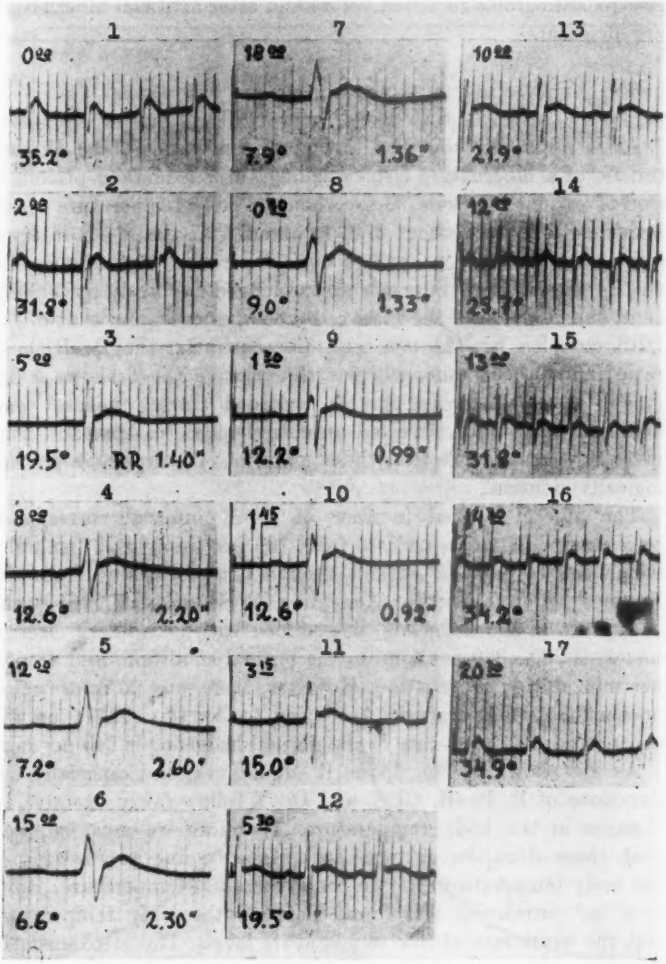

Fig. 2. Serial records (Lead II) obtained from a hedgehog in different stages of artificial hibernation. Body weight 460 g. The hedgehog has received an insulin injection (2 I. U.) and has been put into the refrigerator, where it has gone into artificial hibernation (2—7). After this it has been taken into room temperature to wake up (8—17). The actual R—R interval, body temperature, and the time which had elapsed since the beginning of the experiment or from the moment when the animal was transferred to room temperature to wake up, appear from the figure. The slow oscillation in tracing 7, which resembles a U wave merging partially with the T wave, is an artifact.

electrocardiograms recorded before and after artificial hibernation are identical.

Discussion.

As is apparent from the foregoing observations, the characteristic of a hibernation electrocardiogram is a marked prolongation of various intervals. Thus, when the body temperature of the hedgehog falls from about 37°C to about 7° C, the R—R interval appears to increase on average by 775 per cent. Simultaneously, the P—R and Q—T intervals are prolonged averagely by 500 per cent, the duration of the P wave by 350 per cent, and that of the QRS complex by 425 per cent. Consequently, the P—R/Q—T ratio remains constant, whereas the relative Q—T shows a decrease. The electrocardiograms recorded during natural and artificial hibernation revealed an entirely identical pattern. This gives further evidence that these conditions of torpor are physiologically identical.

The aforementioned increase of R—R interval corresponds to a change in the heart rate from 188 per min. to 22 per min., which differs to some extent, though not essentially, from the findings reported before. According to BUCHANAN (1911) the heart rate in the hedgehog, determined with a capillary electrometer, is 280—320 per min. in the normal condition, and 48—70 per min. during hibernation. However, there was no information on the body temperature. According to ARPINO (1937), on the other hand, the heart rate of the normal hedgehog is 240 per min.

As was shown in Fig. 3, the R—R interval and especially the durations of P, P—R, QRS, and Q—T follow fairly exactly the changes in the body temperature. Therefore we must suppose that these duration changes are primarily due to changes in the body temperature, *i. e.* in the myocardial temperature. However, no correlation was found between the body temperature and the heart rate at low temperature levels. The mechanism of this phenomenon is not clear. There is evidence, however, that it is at least partially due to fluctuations in the carbon dioxide content of the blood, a sequel of the Cheyne-Stokes-like breathing known to occur in hibernating mammals. This interpretation is also in agreement with the observation reported by LYMAN (1951) that the effect of increased CO_2 on the respiration and heart rate of hibernating golden hamsters (*Mesocricetus auratus*) and

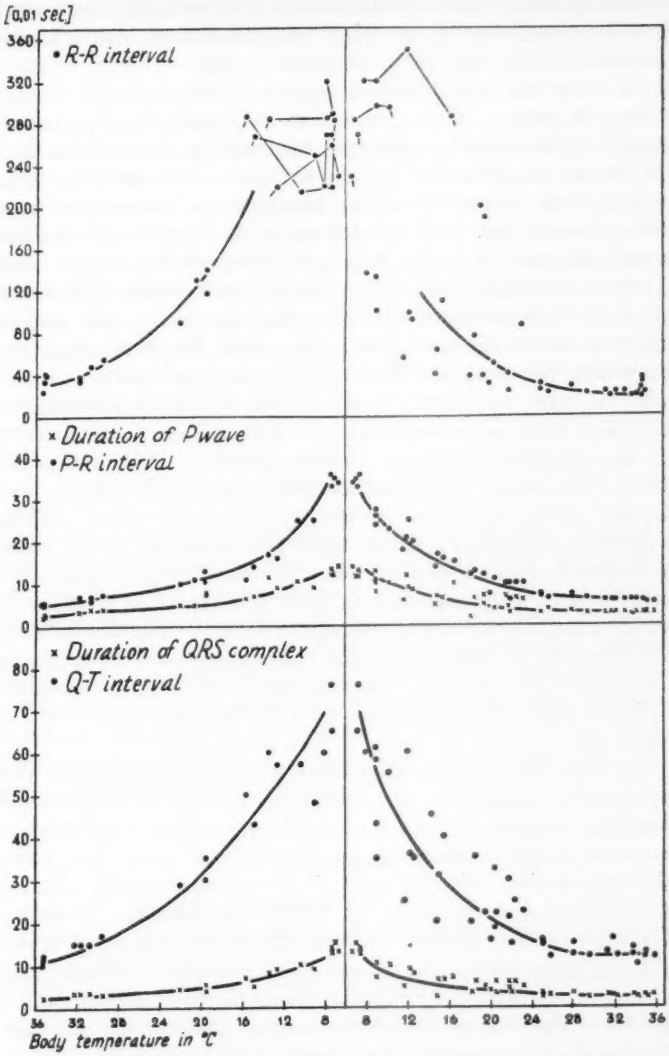

Fig. 3. The relationship of body temperature to the durations of R—R, P, P—R, QRS, and Q—T in different stages of artificial hibernation (four hedgehogs). Durations are expressed in 0.01 sec., body temperature in °C.

ground squirrels (*Citellus tridecemlineatus*) is variable in intensity. Moreover, variations in the vagal tone, associated with separate respiratory excursions, may contribute to this.

The fact that the durations show a tendency to be shorter during the process of awakening can be explained as due to an altered difference in temperature between the myocardium and the ventro-caudal surface of the abdomen, on which the temperature was measured in this investigation. For it has been demonstrated that during awakening the heart and thoracic region are warmed faster than any other portion of the body (LYMAN and CHATFIELD 1950, SUOMALAINEN 1953). Alterations in various physiological equilibria may also play a part, particularly as far as the heart rate is concerned. Many years ago the hypothesis was presented that the process of awakening is essentially a mass activation of the centers of the hypothalamus, becoming manifest, among others, as maximal functional activity of the sympatheticoadrenal system (BRITTON 1928, BARCROFT 1934). The recent investigations performed by CHATFIELD and LYMAN (1950) have given further support to the validity of this hypothesis. The electrocardiographic data presented in this paper also seem to furnish evidence for this view. The abnormally fast heart rate in hedgehogs waking from hibernation is in all probability a manifestation of increased sympathetic activity, probably accompanied by certain humoral components, such as increased contents of nor-adrenalin, adrenalin and lactic acid in the blood, which in their turn contribute to a further acceleration of the heart rate.

The fact that the duration changes are not associated with noteworthy changes in the voltage of various deflections and segments suggests that the process of depolarization and repolarization is evenly retarded, in a "physiological" way, throughout the myocardium without any electrocardiographically demonstrable focal disturbance in the impulse propagation. Therefore, the total systolic ejection phase is apparently prolonged, which is reflected in lowered blood and pulse pressure, a premise confirmed by the author's preliminary investigations on the carotid pressure. These showed that the carotid pressure in hedgehogs, measured with a mercury manometer, is normally about 115/110 mm Hg, and at a body temperature level of 13° C in mean 35/35 mm Hg.

The electrocardiographic changes in the hibernating hedgehog.

reported in this study, are approximately in agreement with those described in other hibernating mammals (cf. for instance HECHT 1915, CHATFIELD and LYMAN 1950, LYMAN 1951). However, the A—V dissociation occurring in golden hamsters early in the process of awakening (CHATFIELD and LYMAN 1950) has never been established in hedgehogs. On the contrary, the electrocardiographic pattern in non-hibernating mammals, in association with experimental hypothermia, differs essentially from that in hibernating animals. Initially the durations are gradually prolonged, as in hedgehogs entering hibernation. But simultaneously a marked deformation of the ventricular complexes appears. This is followed, usually below a body temperature of 20° C, by ventricular ectopic beats and ventricular fibrillation (cf. for instance BIGELOW and co-workers 1950), probably reflecting intrinsic myocardial damage.

Summary.

Electrocardiographic changes in the hedgehog, *Erinaceus europaeus L.*, associated with natural and artificial hibernation, are reported. The two conditions revealed an identical electrocardiographic pattern, consisting principally of marked prolongation of various waves and intervals. No noteworthy voltage changes were found. The changes described are most likely due to the lowered body temperature. Other possible mechanisms are discussed. In addition, the evident type-difference of the electrocardiographic patterns in hibernating and hypothermic non-hibernating mammals is stressed.

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The Transport of Urine in the Upper Urinary Tract.

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Previous attempts to elucidate the function of the upper urinary tract as "dead space" in clearance experiments have so far merely been concerned with measurements of the so-called appearance time (a. t.) at different diuresis (SHANNON et al. 1941, MORALES et al. 1950, SMITH et al. 1938, MC SWINEY and WARNENER 1950). The a. t. is the time elapsed from the moment a dye is injected intravenously to the moment it appears in the bladder. This time interval, corrected for the circulation time for the dye from the vein to the kidney (corrected a. t.) will therefore indicate the time used for the fastest particles to travel from the glomerulus to the end of the ureter. As the only way of getting information about the magnitude of the dead space, this figure is obviously insufficient as it is known from X-Ray studies that intense mixing is continuously going on in the pelvis, and the *average* delay therefore at least in this part has to be very different from the delay of the fastest particles. A method has been devised for measuring the average delay in the whole upper urinary tract, and it will be shown, how this and the corrected a. t. are able to give all information necessary to evaluate the magnitude of the fluid content in the different parts of the upper urinary tract, and how to correct for "dead space" error in many types of clearance experiments and other experiments where this volume plays a rôle: Determinations of extracellular space by means of Inulin (KRUHÖFFER 1946, GAUDINO and LEWITT 1949 and BERGER et al. 1950) and when the specific activity of phosphate in plasma is compared with that in urine (GOVAERTS 1948, HANDLER and COHN 1951 and STRÖM 1950).

The Working Hypothesis.

As a hydrostatic pressure gradient is responsible for the transport of fluid through the tubules (WINTON 1937), this flow would be of the laminar type if the possibility of other influences could be excluded. However, re-absorption of water and dissolved substances is going on throughout the proximal and distal tubules and consequently transversal concentration gradients will arise and result in a more uniform velocity for all particles entering the tubule. In the collecting tubules the joining of streams presumably is working in the same direction by disturbing the laminar flow and consequently no big difference between velocities of the fastest and the slowest particles is to be expected.

Series of rapid X-Ray pictures have given detailed qualitative information concerning the function of pelvis and ureter (FUCHS 1932, HERBST 1937, MARSHALL and SHANKS 1938, NARATH 1940, BEGG 1946 and JOHNSON 1951). When the diuresis is low (below a few ml/min.), this part of the urinary tract is working in a contracted position ("Engstellung" of FUCHS 1932). The urine entering the pelvis is continuously mixed, and once in a while a peristaltic wave will propel a spindle of urine through the otherwise closed ureter to the bladder. These spindle seem to proceed at a very fast rate, a few seconds through the whole ureter. At higher rates of urinary flow, this pattern is somewhat changed. The pelvis is still mixing the urine vividly but is working with a higher maintenance filling (the "Weitstellung" of FUCHS 1932), and the ureter is filled all the time and divided into 3 cysts with functional sphincters and detrusors. The volume of these is always small in comparison with the volume in the pelvis.

These considerations have led to the following working hypothesis: The transport through the tubules and the ureter will delay all filtered particles in a fairly uniform way and these parts will consequently not contribute considerably to the magnitude of the mixing pool in the urinary tract. On the other hand, the rapid mixing in the pelvis will carry the fastest particles so rapidly through this pool that they will not be delayed in this part of the urinary tract. Consequently the corrected a. t. should indicate the average delay in the renal tubules and ureter, and the "pool" of the urinary tract should be identical with the volume of the pelvis.

Experimental Approach.

1. The Tubular Delay.

The relationship to the diuresis: When at low diuresis the upper urinary tract is working in "Engstellung" the ureter will contribute to a. t. only as a consequence of the intermittent activity, because the urine spindle travels very fast. In order to measure the frequency of this activity at different diuresis, together with the a. t., the following experiments were carried out.

Methods: Two trained female unanaesthetized dogs, about 18 kg body weight, and 100 g kidney weight, were placed on their side on a table with loosely fixed hind limbs. The bladder was catheterized with a plastic catheter with several holes at the tip and with a fine plastic tube fixed to the side and ending at the very tip of the catheter. Through this tube a continuous flow of a 2 % Na_2HPO_4 aqueous solution was injected at a rate of about 20 ml/min. In addition to this water flow, bubbles of air were led to the bladder through the same narrow plastic tube. The flow out of the bladder through the catheter was led to a flask connected with a water suction pump to keep the contents under a slight vacuum. In this way the bladder was kept contracted around the tip of the catheter, and the flow out was not continuous but broken into segments in the catheter by means of the air bubbles. Phenol red was injected intravenously and the moment of arrival of the color in the bladder was recorded (a. t.). Then the frequency of color waves was determined for 2—5 minute periods. As a few experiments showed that inulin and phenol red had identical a. t., the last was used exclusively.

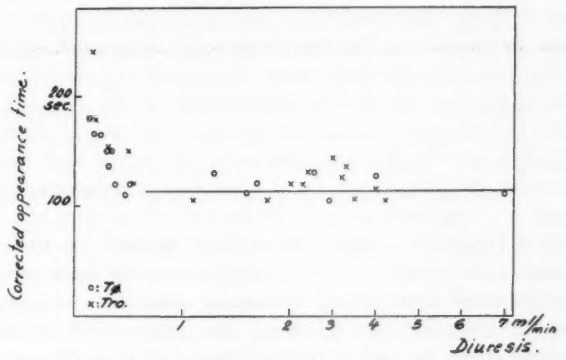

Fig. 1. The relationship between the corrected appearance time and the diuresis for two dogs. The scale for the diuresis interval 0—2 ml/min. is twice as big as for higher diuresis.

Results: The relationship between the corrected a. t. and the urine flow/min. is shown in fig. 1. From the recorded a. t. is subtracted 10 sec. (the circulation time from the vein to the kidney) to get the corrected a. t. It will be seen that at diuresis above 0.5 ml/min. the corrected a. t. is independent of the diuresis, and below this the a. t. varies inversely with the diuresis.

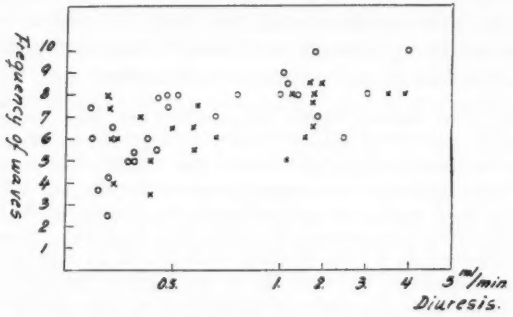

Fig. 2. For the two dogs is shown the relationship between the frequency of the peristalsis in two ureters and the diuresis in ml/min. The abscissa has the same scale as in fig. 1.

In fig. 2 the relation between the frequency for arrival of color waves to the bladder and the diuresis is shown. It will be seen that this frequency is rather independent of the diuresis. In a recent paper MORALES et al. (1952) have published results which conform with my observations on this point. As it is most likely that the peristalsis of the two ureters is independent of each other, fig. 2 shows that about every 10 seconds a peristaltic wave proceeds from the one or the other pelvis to the bladder, and therefore the average time interval between the arrival of the dye at the pelvis and the following peristaltic wave will be 5 seconds. The average delay induced by the tubules is consequently the corrected a. t. minus 5 seconds. A fact relevant from the point of view of clearance experiments is that pressure on the abdomen was rapidly followed by a color wave. As application of such a pressure is the usual procedure used for emptying the bladder in clearance experiments, the ureter will contribute nothing at all to the delay of the fastest particles. Therefore in clearance experiments the simple delay as measured by the corrected a. t. is exclusively caused by the tubules.

The relationship to the glomerular filtration rate: The influence of the filtration rate on the tubular delay was investigated by measuring the a. t. in unanaesthetized female dogs, 20 kg body weight over a period of 1—2 years. The changes in the filtration rate were spontaneous, presumably caused by changes in the diet. The diuresis was increased beyond 1.7 ml per min. by means of hypotonic saline injected intravenously. As fig. 1 showed, the a. t. is then independent of the diuresis, and in addition it is then not necessary to wash the bladder with a continuous flow to get an accurate determination of a. t. Fig. 3 gives the rela-

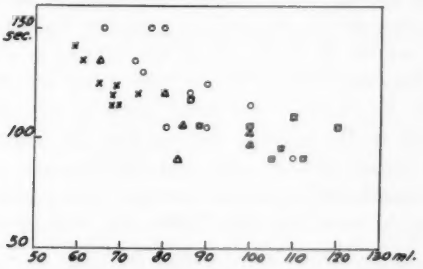

Fig. 3. The relationship between the corrected a. t. and the filtration rate for four dogs. The diuresis was bigger than 1.7 ml/min. for all the determinations.

× Tr. body weight 18 kg, kidney weight 110 g
△ Va. » » 24 » » » 150 »
○ Po. » » 22 » » » 112 »
□ B. » » 26 » » » 200 »

tionship between the glomerular filtration rate and the corrected a. t. (here identical with the tubular delay as an intermittent light pressure on the abdomen eliminates the delay in the ureter). The following correlation coefficients were found: 0.74 ($P = 0.03$), 0.66 ($P = 0.05$), 0.61 ($P = 0.08$) and 0.94 ($P = 0.01$). Consequently the tubular delay is inversely related to the glomerular filtration rate for spontaneous variations in this rate.

Discussion: The previous investigations on a. t. at different urine flows have all given results very similar to fig. 1 as well in dog (MORALES et al. 1950) as in man (MC SWINEY and WARNENER 1950). The only difference between dog and man in this respect is that the diuresis beyond which a. t. is independent of the magnitude of the diuresis is 0.5 ml/min. in dogs but about 2 ml/min. in man. If the hypothesis suggested here is essentially true, this rela-

tionship between diuresis and a. t. will indicate that the delay in the collecting tubules of the final urine only plays a significant rôle when the diuresis is below these limits. If the delay in the reabsorbing parts of the tubules is independent of the diuresis and the volume of the lumen of the collecting tubules is independent of the diuresis when the diuresis is below 0.5 ml/min. in dogs, the curve in fig. 1 may be used to evaluate the order of magnitude of the volume of the collecting tubules. When for instance the diuresis is 0.2 ml/min. the tubular delay is about 60 seconds more than the average value for the delay at diuresis larger than 1 ml/min. and it may consequently be argued that it takes 60 seconds for 0.2 ml to pass the collecting tubules, because the volume of that part of the tubules is about 0.2 ml. At diureses between 0.5—1 ml/min. the delay should, according to this value, be of about the same order of magnitude as the error involved in the present method for the determination of the tubular delay (20—12 seconds) and should consequently not deviate significantly from the average value for large diuresis. When calculated from histological data the volume of the collecting tubules is of the order of magnitude 1/3—1/2 ml for dogs (SPERBER 1953).

It seems a striking fact that the "augmentation limit" for urea in man (MÖLLER et al. 1929) and in dog (SHANNON 1936) coincides with the diuresis below which the diuresis begins to play a perceivable rôle for the delay in the collecting tubules. It seems quite understandable that the back diffusion of urea from the urine is dependent on the time during which the urine is in contact with the collecting tubules. This is in accordance with the conclusion of EFFERSØE (1952) that the significance of the diuresis for the urea loss in the tubules can not be attributed merely to the differences in the concentration index.

II. The Pool in the Upper Urinary Tract.

When the concentration in the urine of a clearance substance is changing from a concentration R_1 in the beginning of the period to the concentration R_2 at the end, and the volume of the pool where continuous mixing is going on is V, then the correct clearance formula is

$$Cl = \frac{M + V(R_2 - R_1)}{t\,P_m} \quad \ldots\ldots\ldots\ldots\ldots \quad (1)$$

where M is the amount of substance collected in the bladder urine in the time interval t in which P_m is the average plasma concentration. In this formula several suppositions are made. An attempt to elucidate the significance of the most important ones for the validity of the formula is given in the following points.

1) The formula when used in practice is only absolutely correct if the change in the concentration R *is* linear, as it is not possible to measure the concentration in the pool itself, but only in a drop running out of a catheter in the bladder. As a pressure on the abdominal wall will result in a peristalsis in the ureter which proceeds at a high speed, the delay in the ureter plays no important rôle. But the catheter may cause a considerable delay at a diuresis below 1 ml/min. If the volume of the catheter is 0.4 ml, then the average delay here will be $\frac{0.4}{\text{Diuresis}}$ minutes. This may cause unpredictable grave errors at low diuresis about 0.1 ml if the changes in the concentrations in the pool are very irregular through the period. However, when the concentration variation is regularly linear through the whole period, then the difference $R_2 - R_1$ will always be measured correctly. By means of a proper schedule for the clearance experiment it seems likely that very irregular variations during the period most often can be avoided.

2) The formula is only correct if V is the same at the beginning and the end of the period. If that is not the case, the correct formula will be

$$Cl = \frac{M + V_2R_2 - V_1R_1}{t\ P_m}$$

V_2 is here the volume at the end and V_1 the volume at the beginning of the period. If the clearance is calculated from formula (1), then the V has to fulfill the following equation

$$V = \frac{V_2R_2 - V_1R_1}{R_2 - R_1} = \frac{V_2}{1 - \frac{R_1}{R_2}} - \frac{V_1 - \frac{R_1}{R_2}}{1 - \frac{R_1}{R_2}}$$

From this it is obvious that if R_1 is big in proportion to R_2, then V is very close to V_1, and if R_1 is very little then V is nearly identical with V_2. But this is only valid if the relative changes in R concentrations are big in proportion to the relative changes in V as seen of the following examples

$\frac{R_1}{R_2}$	5					1.5						
V_1	1	2	3	4	5	1	2	2.5	3	3.5	4	5
V_2	5	4	3	2	1	5	4	3.5	3	2.5	2	1
V	0	1.5	3	4.5	6	—7	—2	0.5	3	5.5	8	13

This illustrates the fact that if the relative changes in R are small, then V has to be rather constant if the formula (1) shall be valid.

3) The formula (1) implies that there exist one V and one set Rs, but in reality there are two renal pelves. Consequently the true formula should be

$$Cl = \frac{M_x + M_y + V_x (R_{x_2} - R_{x_1}) + V_y (R_{y_2} - R_{y_1})}{t \cdot p_m}$$

In this formula M_x and M_y are the collected amounts of substance secreted from each side where the volumes are V_x and V_y and the initial and final concentrations of the clearance substance R_{x_1}, R_{x_2}, R_{y_1} and R_{y_2}. If the formula (1) shall give the right value for Cl, then the following equation must be fulfilled for the initial and the final concentrations:

$$R = \frac{V_x R_x + V_y R_y}{V_x + V_y} = R_x + (R_y - R_x) \frac{1}{1 + \frac{V_x}{V_y}}$$

However, not only may the concentrations and the volumes of the pelvis be different on each side, but the partial volume from each side that contributes to the small samples in which the initial and final concentrations of clearance substance in the urine is measured may be different. If this volume from the x-side is a and from the y-side b, then the actual concentration measured is

$$C = \frac{R_x a + R_y b}{a + b} = R_x + (R_y - R_x) \frac{1}{1 + \frac{a}{b}}$$

Consequently the percentage difference between the measured concentration C and the concentration R (where R appears in formula (1) for true clearance, and $V = V_x + V_y$), is:

$$\frac{100\ (R-C)}{R} = 100\ \frac{\frac{1}{1 + \frac{V_x}{V_y}} - \frac{1}{1 + \frac{a}{b}}}{\frac{1}{\frac{R_y}{R_x} - 1} + \frac{1}{1 + \frac{V_x}{V_y}}}$$

This difference is shown in fig. 4 in relation to different values of the three ratios in the equation. Of course it is impossible to know what values these ratios may have in different cases. However, as far as the ratio between the concentration of the substance on both sides is concerned experiments with catheterisation of the two ureters of normal dogs have always shown nearly identical concentrations on the two sides (Blake et al. 1949, Hall and Selkurt 1951, Kaplan

et al. 1951 and GOODEYER et al. 1951). The difference on the two sides is very seldom bigger than 20 per cent. It will be seen from fig. 4 that with this difference between R_y and R_x, the pelvis on one side may be 5 times as big as that on the other, and one side may contribute 10 times as much as the other side and yet $100\,\frac{R - C}{R}$ will not exceed 14 per cent. As the concentration is measured in a sample collected just after application of a light pressure on the abdomen, and this is known to cause a peristaltic wave in the ureter, it seems not likely that the contribution from one of the pelves is more than 10-fold the contribution from the other. Nor is it likely that one pelvis is more than 5 times as big as the other.

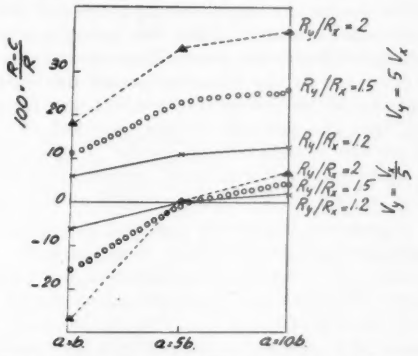

Fig. 4. The ordinate is the percentage difference between the actually measured concentration C (page 46) and the R in formula (1) and (2) when $V = V_y + X_x$. The abscissa is the ratio between the partial volumes of urine from the two sides, contributing to the sample in which C is measured. This relation is shown for different ratios of the concentrations on the two sides (R_y/R_x) and these relationships for two ratios of the pelvis volumes on the two sides (V_y/V_x).

Of these three suppositions involved in formula (1) point 2 is the most important for all levels of diuresis. When the diuresis is below 0.5 ml/min. the supposition mentioned as point 1 will be of significance for the validity of the formula. The smaller the diuresis, the greater the significance is.

The formula (1) has been used for an indirect determination of V. This V must be independent of the clearance substance used, and consequently the following equation has to be fullfilled for the two substances creatinine and inulin because in dogs they have identical clearances:

$$\frac{M_i + V(R_{i_2} - R_{i_1})}{t \cdot p_{i_m}} = \frac{M_c + V(R_{c_2} - R_{c_1})}{t \cdot p_{c_m}} \quad \ldots\ldots (2)$$

In this equation M_i, R_i and p_i are the values for inulin in formula (1) and M_c, R_c and p_c the values for creatinine.

Methods: The use of formula (1) has made the clearance procedure somewhat different from the usual. Consequently it is necessary to go into details in the description.

Urine collection: A catheter of rubber with several holes was left indwelling in the bladder. It was just long enough to reach outside the perineum and had a volume of about 0.4 ml. In order to collect the urine it was connected with a 20 cm long rubber tube hanging down in a vial, with the tip some 10 cm below the tip of the catheter. The suction applied on the bladder contents in this way was sufficient to keep the bladder practically empty during the period when the diuresis was smaller than 3—4 ml/min. That this actually was the case was assured by using colored wash water. The amount of urine necessary to wash the color out in the following period was very seldom more than the capacity of the catheter could account for. This together with the fact, that the amount of fluid collected at a washing very seldom exceeded the amount of wash water + the urine flow during the washing procedure (half a minute), showed that the bladder was well contracted around the tip of the catheter all the time.

Analytical procedures: Inulin was analysed according to a procedure previously published (Bojesen 1952). Sometimes the same protein-filtrate as used for the creatinine determination was used for the inulin determinations. Creatinine was analysed according to Bonsnes and Tausky (1945), or Peters and Van Slyke (1932). It was necessary in each experiment to determine the recovery for creatinine in plasma as it was not possible to find any protein precipitating agent yielding a 100 per cent recovery. In most experiments the method of Steiner et al. 1932 was used. The recovery was checked for two concentrations. The color was read in a Beckmann spectrophotometer model D. U. with permanent cooling arrangement at 520 μ or 530 μ. As the highest possible accuracy was desired, the plasma blank was determined as the difference between the total Jaffe-positive material in the plasma filtrate before injection of creatinine and the level of the endogenous creatinine. The endogenous creatinine was determined in a period before the actual experiment. It was calculated as the plasma concentration giving the same clearance as inulin. The supposition made was, that Jaffe-positive material in urine is creatinine with a clearance identical with that of inulin.

Calculation of the mean plasma concentrations: The plasma concentration corresponding to the amount of clearance substance collected from the bladder plus the increase or minus the decrease of the substance in the pelvis during the period can be found simply by antedating the moments for the emptying of the bladder by a time interval corresponding to the tubular delay. As the tubular delay is dependent on the diuresis (fig. 1) it is necessary to know the level of diuresis at the end and beginning of the period. When the plasma curve is linear in

the period, the mean plasma concentration is the concentration in the middle of the period transposed in the aforementioned way. If the type of the plasma curve is logarithmic, the formula

$$p_m = \frac{p_2 - p_1}{2.303 \ (\log p_2 - \log p_1)}$$

was used. Here p_2 and p_1 are the concentrations at the end and beginning of the transposed period. When the plasma curve was rising very steeply, it mostly was following a parabolic curve and the plasma mean was calculated according to the formula

$$p_m = 1/6 \ (p_1 + 4 \ p_2 + p_3) + 1/3 \ (p_1 - p_3) \ \frac{t_1 - t_2}{t}$$

in which p_1, p_2 and p_3 are the concentrations of the substance in the plasma at the start of the period, near the middle, and at the end, and in which t is the time interval between p_1 and p_3, t_1 the time interval between p_1 and p_2 and t_2 the time interval between p_2 and p_3.

The procedure for an experiment: Unanaesthetized, trained female dogs, body weight about 20 kg, were placed on their side on a table with their hind limbs loosely fixed. A cannula was plunged into the femoral artery, and a continuous flow of heparinized blood kept the cannula open. Inulin was injected as a priming dose and a continuous injection from a motor driven syringe is started. Usually the level of inulin was 20—30 mg %. 15—20 minutes later the endogenous level of creatinine was determined during a short period. Then creatinine was injected intravenously in the same way as inulin. After 20—30 minutes two control periods were started to see how close to identity the two clearances were. Then the infusion of both substances was disrupted and injection of a very concentrated solution of inulin was started. After two periods of about 8 minutes each, with increasing concentration of inulin and decreasing concentration of creatinine, the inulin injection was disrupted, and injection of a concentrated solution of creatinine was started. This was then followed by two periods of about 8 minutes each.

A clearance period was started by inducing a contraction of the bladder by light pressure on the abdomen. A blood sample (5 ml) from the artery was collected in a tube with heparin, and a drop of urine was collected in a small vial from the tip of the catheter just outside perineum (R_1). The 20 cm rubber tube was connected with the catheter, and injected with about 10 ml colored distilled water, and a bladder contraction was again induced by a light pressure on the abdomen. This wash water was discarded. After some 8 minutes, another drop from the end of the catheter was collected (R_2), a blood sample was taken from the artery and the bladder was again washed with 10 ml water. This final wash water was collected in the same graduate as the urine flowing out of the bladder during the period, and the urine flow was determined by subtracting 10 ml from the total volume. From the two small drops was pipetted 0.1 or 0.01 ml in a 10 ml volu-

metric flask and the rest of the drop together with the content of the graduate was poured in a 100 ml volumetric flask. The concentrations of inulin and creatinine in these volumetric flasks and blood samples were determined, and gave the M, R and p in the formula (2).

The theoretical error in the determination of V from formula (2): The significance of the suppositions involved in formula (2) mentioned previously as point 1, 2 and 3 (page 45—47) is minimized when the change in R concentration is big in the period; also analytical errors in the clearance determinations are minimized in this way. It is possible to compare the analytical errors in the clearance determinations with the error in the determination of p_m introduced from the inaccuracy in the evaluation of the tubular delay in the following way. Later on (page 57) it will be shown that the average delay in the pool in the pelvis of molecules entering the pool is about V/D min. (D is the diuresis in ml/min.) for fairly constant diuresis. It can be deduced from the scatter in fig. 1 that the tubular delay is determined with an error of 12 seconds = 1/5 min. This error in the antedating will give an error in the determination of V which again will give an error in V/D of 1/5 min. As 1 ml pool will give a difference in the two clearances (as calculated from the usual clearance formula: $Cl = \frac{M}{t_{p_m}}$) that amounts to

$$\left\{ \frac{R_{i_2} - R_{i_1}}{t\ p_{i_m}} - \frac{R_{c_2} - R_{c_1}}{t\ p_{c_m}} \right\}$$

(compare formula (2)), an error in V of D/5 ml will give a difference in the two clearances of

$$\frac{D}{5} \left\{ \frac{R_{i_2} - R_{i_1}}{t\ p_{i_m}} - \frac{R_{c_2} - R_{c_1}}{t\ p_{c_m}} \right\} = F$$

This figure F is therefore the error in the determination of V by means of the formula (2) caused by an error in the evaluation of the tubular delay of 12 seconds.

In the control periods the difference between the creatinine clearances and the inulin clearances has been found to be -0.4 ± 2.8 ml/min. The error on this difference may be taken as a measure of the analytical error on the determination of the difference between the two clearances. The larger the variations that are obtained in plasma concentrations the larger is the difference in the clearances caused by a certain volume V at a certain diuresis, and the smaller is the significance of the analytical error on the determination of the difference between the two clearances. It is, however, of course not possible in this way to diminish the error on the determination of V caused by the error of 12 seconds on the tubular delay, as this error (F) is proportional to the difference between the two clearances.

If F for a certain period is smaller than the analytical error on the determination of the difference in the clearances (2.8 ml/min.), this analytical error will limit the accuracy of the determination of V. For this reason it has been endeavoured to obtain as large variations in

the plasma concentrations as practically feasible. In many periods it was achieved to make F larger than the analytical error. In such periods this error has not limited the accuracy of the determination of V, and the accuracy has been close to D/5, which is the highest obtainable accuracy when the uncertainty on the tubular delay is 1/5 min. When the two errors on the determination of V are equal, and consequently both are $\pm$ D/5 when expressed in ml, the uncertainty on the determination of V caused by the combined errors will be $\pm$ D/5 $\cdot \sqrt{2}$ ml. In periods in which F is smaller than 2.8 ml, the analytical error will predominate and the uncertainty will be larger than D/5 $\cdot \sqrt{2}$ ml.

Though F is an error on the determination of V it is an advantage for the determinations, that F is larger than the analytical error, as F at a certain diuresis is a percentage error on the determination of the difference of the two clearances underlaying the determination of V, whereas the analytical error presumably has a fairly constant absolute value.

Results: In fig. 5 are shown the plasma curves for the two substances in relation to the moments for emptying the bladder, here indicated by arrows. It is shown how the correction for the tubular delay is carried out, by antedating the moments for bladder washing. As the clearances for both substances are independent of the level in the plasma, the conventional concentrations values are not calculated, but the extinction values are given directly. In table 1 the clearances calculated according to the usual formula $Cl = \frac{M}{t\, p_m}$ are given together with the values of V calculated according to the formula (2), and the diuresis for the period.

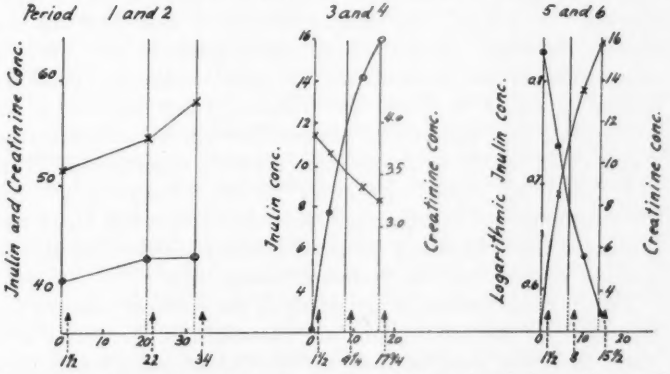

Fig. 5. The plasma curves for inulin o and creatinine × in relation to the actual moments for bladder washing (arrows) in an experiment for indirect determination of the volume of the pool in the upper urinary tract. The intervals between the three series were usually smaller than 15 minutes.

Table I.

Data from the same experiment as shown in fig. 5. "Inulin clearance" and "creatinine clearance" are the clearances calculated according to the conventional formula, with an a. t. correction as the only correction.

	Control					
	1st period	2nd period	3rd period	4th period	5th period	6th period
"Inulin clearance"	89.5 ml/min.	87.6 ml/min.	69 ml/min.	95 ml/min	111 ml/min.	93.4 ml/m
"Creatinine clearance".	89.6 »	88.2 »	81.5 »	103 »	81.4 »	100.0 »
Diuresis	0.8 »	0.95 »	0.7 »	0.8 »	1.6 »	1.7 »
V	?	?	0.97 ml	1.0 ml	1.1 ml	1.4 ml

In this experiment the 4th and the 6th periods gave the same results as the 3rd and 5th in spite of the fact, that the relative changes in the concentrations are very much larger in 3rd and 5th than in 4th and 6th. In the periods 3 and 5 the figure F mentioned on page 50 was bigger than the analytical errors in the determination of the differences in clearances (2.8 ml) and consequently the analytical error involved in the determination of V was smaller than D/5 ml and the total error only slightly larger than D/5 ml. In the periods 4 and 6 the relative changes in R concentrations were too small to obtain this accuracy.

In fig. 6 is shown the relationship between the diuresis and the volume V. In diagram a and b all the values for V are determined with an accuracy of about D/5 ml. But in c and d F had a value between 1.4—2.8 ml. The diagrams in fig. 6 show that for low diuresis, between 1.5 and 0.2 ml, the volume of the pelvis is independent of the diuresis, and the same is the case when the diuresis is above 4—5 ml. The volume at low diuresis is approximately 1 ml and at high diuresis different for different dogs, 7 and 12 ml for the two dogs most carefully investigated. These two functional states correspond to the "Engstellung" and "Weitstellung" of the German radiologists. Between these two functional states there is a linear relationship between the diuresis and the volume, but the scatter is rather high.

The maximal volume of the pelvis in the removed kidneys was measured as the amount of fluid contained in the pelvis after water had been injected from ureter without considerable pressure. The "dead volume" was measured in this way for several dogs. These volumes were always somewhat larger than the maxi-

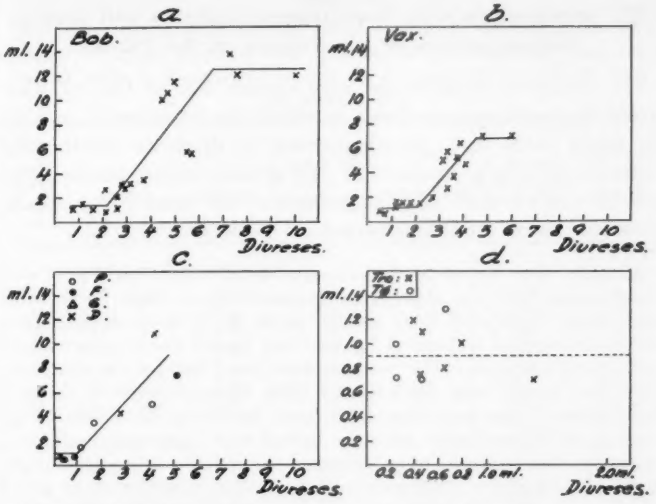

Fig. 6. The relation between the diuresis in ml/min. and the indirectly determined pelvis volumes. The kidney weight and the maximal volumes of the pelves after kidney removed were respectively in a: 200 g and 15.5 ml, and in b: 150 g and 14 ml. In c a few data are given for 4 different dogs. In d the diuresis interval 0.2 to 1 ml is more carefully investigated on two dogs.

mal volumes of the living pelves. For 10 dogs with body weight from 19 to 25 kg and kidney weight between 45 to 107 g for each kidney no relationship was found between the maximal volumes of pelves and kidney weight or body weight. Therefore, this volume must be determined for each dog by the indirect method whenever accurate data are desired (see "Implications").

In a preliminary report (BOJESEN 1949) some very low figures for V were found when the diuresis was increasing rather steeply during the period. These peculiar results can be explained straightforwardly from the suppositions that were used in deriving formula (1) and mentioned as point 2 (page 45), because in these early experiments the concentrations of the substances were not changed enough to eliminate the influence of changes in the volume V on the validity of the formula (1). As these small volumes were never seen, when the plasma concentrations were changed as much as to make the F (page 50) larger than 1.4, it seems most probable that they are artifacts.

III. Experiments with Simultaneous Indirect and Direct Determinations of the Volume of the Pelvis.

The following experiments were carried out to test to what extent the working hypothesis in which the formulas (1) and (2) are based holds true. In other words to illustrate whether the corrected a. t. is a measure of the average delay in the renal tubules and whether only the volume of the renal pelvis constitutes the "pool" of the urinary tract (V in formula (1)).

Methods: Two types of experiments were carried out, one with anaesthetized and the other with unanaesthetized dogs. In the first ones female dogs with body weight about 20 kg were anaesthetized with pentobarbital sodium. A ligature was placed loosely around each renal artery and ureter. The two ends were stung through the abdominal wall. The wound was stitched and after determination of the a. t. the volume of the pool was determined indirectly by means of the formula (2). Immediately after the period with increasing inulin concentration the ligatures were tightened around the artery and ureter of one of the kidneys. Then another indirect determination of the pool V was carried out just after an a. t. determination and the ligatures around the artery and ureter of the remaining kidney was tightened. The kidneys were removed and the contents of the two pelves were determined by injection of 1 ml 1 % diodrast through a cannula in the ureter near the pelvis. From the dilution of diodrast the actual contents of pelvis were calculated (the direct determination).

The other procedure was carried out on unanaesthetized dogs. After determination of a. t. and an indirect determination of the volume of the pool V the dog was killed immediately after the last bladder washing by 220 volts a. c. with one electrode in the mouth and the other in the rectum. The blood pressure drops immediately. As fast as possible (1—2 min.) the abdominal wall was opened and clamps were placed on ureter and urethra, and the kidneys with the urinary tracts were removed, the contents of the renal pelvis and the bladder were determined by the dilution technique. By analysis of the creatinine contents in the bladder and the concentration in the last urine sample collected just before the washing, the amount of urine in the volume of fluid found in the bladder was calculated. The difference between the total volume and the volume of urine was wash water remaining in the bladder after the last washing. The urine thus found in the bladder was contents from the pelves transported from the pelves to the bladder in the few minutes elapsed from the moment the blood pressure drops, to the moment the clamps were placed on the ureters. It was not urine left over from the last clearance period. This can be deduced from the following facts. The total volume from the washing was never more than 130 % of the volume of the wash water, and the amount of fluid found in the bladder by the aforementioned technique was only around 0.5 ml. That means that the largest possible amount

Table 3.

Nine experiments with as well direct as indirect determinations of the pool in the renal pelvis in dogs. The indirectly determined pool size on the left sides in experiment 1a and 2a is calculated as half the total V in formula (2).

Experiment number	Side of pelvis	Diuresis for two kidneys ml/min.	Filtration rates for two kidneys ml/min.	V indirectly determined ml	V directly determined ml	Maximal pelvis volume ml
Anaesthetized and operated dogs.						
1a	left side	0.6	60	0.8	0.5	5
1b	right »	0.7	56	2.8	3.0	6
2a	left »	0.35	48	1.4	1.5	4
2b	right »	0.60	52	0.15	0.5	5
3	right »	1.40	100	4.3	3.8	6—7
4	left+right	1.40	72	1.6	2.4	14
5*	left+right	2.0	140	8.0	9.2	15.5
Unanaesthetized dogs.						
6*	left+right	1.4	76	1.4	2.1	8.0
7*	left+right	0.65	53	1.3	0.95	9.0
8*	left+right	2.40	68	4.0	3.0	7.0
9*	left+right	0.60	28	—0.1	+0.2	5.0

of urine left in the bladder from the previous period was about 0.1 ml. The amount of urine in the bladder and in the two removed pelves was therefore the contents of the pelves at the moment the blood pressure drops.

Results: The results are given in table 3. As far as the accuracy of the indirect determinations is concerned experiments marked with (*) have the same value of F as the experiments of fig. 6 a and b. In other words the error in the determination of V is approximately (diuresis/4) — (diuresis/5) ml. In the others (experiment 1, 2, 3 and 4) this error is a little larger, around (diuresis/2) ml. As (V/D) in experiment 1 a is $2\,^2/_3$ min., in experiment 1 b: 8 min., in experiment 2 a: 8 min. and in experiment 3: 6 min., this error of $^1/_2$ min. does not invalidate the determination of V. In no. 2 b (V/D) is $^1/_2$ min. and consequently V is poorly determinated; it may well have been 2 times as big. In the experiment no. 4 the accuracy of the indirect determination of V has been about D/3 and with a diuresis 1.4 ml the error is approximately 0.5 ml, and consequently the error in the indirect determination alone will nearly account for the difference between the two values for V determined directly and indirectly. The differences between the direct and the indirect determinations of the pelvis volume

never exceed what the already mentioned errors in the indirect determination may account for, and as that is the case for so different values of the volume of the pool, these experiments seem to show that the working hypothesis is mainly correct.

Implications.

Clearance corrections: The results can be used for correcting the clearance for "dead space" error in the following way:

1) Correction when the diuresis is fairly constant in the period: At constant diuresis it is possible to calculate the average delay caused by the pool in the following way when the type of plasma curve is known.

If the volume of the pool is V, the amount of substance entering the pool per min. $Cl \cdot p(t)$ (the renal clearance times the plasma concentration at the time t, which for a small time interval (dt) may be considered constant), the concentration of the substance in the fluid leaving the pool R(t) and the volume of the fluid leaving the pool per min. D, then the following equation should be right if the fluid in the pool is perfectly mixed

$$V \frac{dR}{dt} = Cl \cdot p(t) - R(t) \cdot D$$

In words: the change per minute of the amount of substance in V is the amount of substance entering per minute minus the amount leaving per minute. If the plasma concentration follows a logarithmic curve according to the formula $p(t) = p(o)\, e^{-ht}$ then by integration

$$R(t) = \frac{p(o)\, Cl}{D - hV} e^{-ht} + Ie^{-\frac{Dt}{V}}$$

For a linear plasma curve the formula is

$$R(t) = \frac{Cl\left(p(o) - a + \frac{aV}{D}\right)}{D} + Ie^{-\frac{Dt}{V}}$$

The significance of the integration constant is that it will define the R(o) for the plasma concentration p(o). If the concentration of the substance in the pelvis is zero at a time (— x), for instance x minutes before the beginning of a clearance period $R(-x) = 0$ and the variation in the concentration of the substance in plasma p(t) from this moment is logarithmic then I is determined by the equation

$$0 = \frac{p(o)\, Cl}{D - hV} e^{+hx} + Ie^{+\frac{Dx}{V}}$$

and consequently

$$R(t) = \frac{p(o)\ Cl}{D-hV} e^{-ht} \left\{ 1 - e^{\left(h - \frac{D}{V}\right)(x+t)} \right\}$$

This formula shows that if $e^{(h-D/V)(x+t)}$ is small in comparison with 1 then the concentration in the urine will follow the formula

$$R(t) = \frac{p(o)\ Cl}{D - hV} e^{-ht}$$

If for instance a 2 per cent error is allowable in evaluation of R(t) then $\left(h - \frac{D}{V}\right)(x+t)$ has to be smaller than -4 as $e^{-4} = 0.018$. If for instance $h = 0.03$ and $D/V = 0.2$ then $x+t$ has to be bigger than 23 min. to obtain that the concentration in the pool corresponds to the plasma concentration with an accuracy of 2 per cent.

If the condition for minimizing the influence of x on R(t) are fulfilled then the equation for

$$R(t) = \frac{p(o)\ Cl}{(D - hV)} e^{-ht}$$

may be used to calculate the average delay of particles running through the pool. This delay may be called τ. By definition

$$Cl = D \frac{R(t)}{p(t-\tau)}$$

and when the R(t) and $p(t-\tau)$ is substituted by the abovementioned expressions

$$Cl = D \frac{p(o)\ Cl\ e^{-ht}}{(D-hV)\ p(o)\, e^{-h(t-\tau)}}$$

and therefore

$$e^{h\tau} = \frac{D - hV}{D} \text{ and } \tau = -\frac{V}{D}\left(1 + \frac{h}{2}\frac{V}{D}\right)$$

For linear plasma curves an analogous derivation gives

$$\tau = -\frac{V}{D}$$

From this description it appears that two conditions have to be fulfilled to make it possible to correct the clearance simply by means of a delay time. 1) The diuresis has to be fairly constant and 2) the plasma concentration must have been changing in the same way as during the period for a certain time (x) before the period in order to make the number $e^{(h-D/V)(x+t)}$ small in relation to 1. When these conditions are fulfilled, the total delay time will be the tubular delay (identical with the corrected a. t. when the significance of ureter is eliminated by light pres-

Table 4.

Determinations of τ by two different methods.

τ determined directly from the total delay time	$\tau = \frac{V}{D}$, and V determined indirectly from the equation (2)	
1 3/4 min.	2 1/4 min.	Unanaesthetized dogs.
1 1/2 »	1 3/4 »	
2 »	2 1/4 »	
1 »	1 »	
7 1/2 min.	8 1/2 min.	Anaesthetized and operated dogs.
8 1/2 »	8 1/2 »	
5 »	6 1/2 »	
1 1/2 »	1 1/10 »	

sure on the abdominal wall) plus V/D (when the plasma curve may be considered linear). For more accurate work the average delay in the pool when the plasma concentrations follows a logarithmic curve should be calculated not as V/D but as V/D $(1 + \frac{h}{2} V/D)$.

In table 4 two groups of consecutive experiments are presented to show how close the average delay in the pool calculated from V/D is to the value found directly by subtracting the a. t. from the total delay time. The total delay time was found as the time necessary to transpose the plasma curves in relation to the actual moments for bladder washing to get identical clearance for the two substances (unilin and creatinine). These two independent determinations of the average delay in the pool seem to give fairly consistent results and consequently render further support for the working hypothesis.

2) Clearance correction when the diuresis is changing considerably in the period: When the diuresis is changing in the period it is necessary to determine the concentrations of the substance in the urine at the beginning and the end of the period (R_1 and R_2 in formula (1)) and to calculate the clearance from the formula (1). When the diuresis is changing within the range of 0.2—1.5 ml the "Engstellung" of the animal is used for V and when the diuresis is changing within a range above 3—4 ml the "Weitstellung" has to be used. In the range between "Engstellung" and "Weitstellung", one single value for V will not give the right clearance and the formula for calculating the clearance

is then $Cl = \frac{M + R_2V_2 - R_1V_1}{t \cdot p(m)}$ and the V_2 and V_1 are judged from the diuresis as calculated from $D = \frac{Cl \cdot p(t)}{R(t)}$ where Cl is the clearance in the period, and p(t) and R(t) are the concentrations in plasma and urine at the end or beginning of the period. From the diuresis at the beginning and the end of the period calculated in this way V_2 and V_1 may be evaluated from the diuresis—pelvis volume relationship for the dog. When the diuresis is smaller than 0.5 ml it is also necessary to take the change in the tubular delay into consideration as the tubular delay at diuresis of this size is dependent upon the diuresis as shown in fig. 1. This is done in the following way. If the diuresis in a ten minutes period increases from 0.2 to 0.5 ml the a. t. decreases about one minute, and an amount of the clearance substance corresponding to a clearance period of 11 minutes is brought to the renal pelvis in the ten minutes period.

When not only the diuresis but also the plasma concentration is changing during the period, the tubular delay corresponding to the diuresis at the beginning resp. at the end of the period must be used in the antedating procedure necessary for the calculation of the mean plasma concentration for the period. The diuresis for these two moments may be evaluated as mentioned before.

The significance of changes in the filtration rate for the clearance correction: As it was found that the tubular delay is dependent not only upon the diuresis but also upon the glomerular filtration rate, the level of this rate should be of some significance for the tubular delay used in antedating procedure in clearance corrections when the plasma concentration is changing. However this uncertainty is included in the error of 12 seconds on the tubular delay and this is usually smaller than the error on the correction caused by the errors on τ.

Just as changes in the diuresis at the low levels (0.2—0.5 ml/min.) also changes in the filtration rate during the period will cause an error on the determination of the duration of the period even if the plasma concentration is not changing. It is of course not possible to use the knowledge about the relationship between the glomerular filtration rate and the tubular delay for such corrections because the filtration rate is not known for the mo-

ments of the end and the beginning of the periods. This is insignificant as far as the error on the determination of the plasma mean is concerned but it may influence significantly the duration of the clearance period. As will be seen in fig. 3, an increase in the filtration rate during a period from 60 ml/min. to 75 ml/min. for the dog Tr. will wash the tubules for a half minute's clearance substance and consequently cause an error on the clearance determination for a 5 minute period of about 10 %.

Corrections when the specific activity of a substance in the urine is compared with that in the plasma: This may be done in just the same manner as was used for two substances with identical clearances but changing plasma concentrations. The active and inactive part of the substance can be considered as two substances with the same clearance. Therefore V or p(m) may be determined, dependent upon which of them is already known.

Some information concerning the function of the tubules gained from the experiments: The experiments in table 3 and the pretty good accordance between the two ways for calculation of τ shown in table 4 indicate that the experimental results are compatible with the working hypothesis. This involves that all tubules have nearly the same average delay, identical with the corrected a. t., and therefore no considerable part of the nephrons can be inactive with an inactive period with a length about the same as the tubular delay or longer. However, as the methods for measuring V are not very accurate, it is not possible to exclude that a small fraction of the nephrons may have inactive periods equal to or longer than the tubular delay. According to table 3 this uniform activity of the nephrons is found even at rather low filtration rates in dogs.

Summary.

A method is devised for measuring indirectly the volume in the renal pelvis in normal dogs. The method is based on the following suppositions: 1) all particles excreted in the urine travel through the tubules at a uniform speed; 2) the renal pelvis is continuously mixing the urine so rapidly that the passage through it does not contribute measurably to the delay of the fastest particles; and 3) that the passage through the ureters will delay the excretion of urine insignificantly when the bladder is emptied by a catheter and light pressure on the abdomen.

The frequency of the peristalsis in the ureter at different levels of diuresis was measured and it was shown that the appearance time for dyes injected intravenously, when corrected for the circulation time and inactive periods of the ureters, is the delay of the particles in the tubules.

The relationship of this tubular delay to diuresis and glomerular filtration rate was determined.

The volume of the renal pelvis determined by the indirect method was found to be dependent on the diuresis in such a way that it is small, about 1 ml for the two sides together, when the diuresis is below 1—1½ ml/min. With increasing diuresis the pelvis volume increases in a linear way until at a diuresis of 4—5 ml/min. a maximal volume is reached. These data are compatible with qualitative X-Ray studies on man.

Some experiments on anaesthetized and unanaesthetized dogs showed that these indirect determinations of the volume of the pool in the pelvis gave results in agreement with the directly found volumes over wide ranges of volumes and diuresis.

The results are used to devise methods for obtaining accurate clearance determinations, even under conditions in which there may be rapid changes in plasma concentration of the clearance substance as well as changes in diuresis.

The results furthermore indicate that the major part of the nephrons are transporting the entering particles at the same speed, and consequently no considerable part of the nephrons has an intermittent activity even at low filtration rates.

The author wishes to express his gratitude to Civilengineer Poul H. Rasmussen for his kind help with some of the mathematical formulations and to Professor Einar Lundsgaard for his great help in the preparation of the manuscript.

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The Effect of Insulin Hypoglycemia on the Secretion of Adrenaline and Noradrenaline from the Suprarenal of Cat.[1]

By

HANS DUNÉR.

Received 9 March 1954.

CANNON, MCIVER and BLISS (1924), using changes in the rate of the denervated heart as a criterion of the secretion from the suprarenal medulla, concluded that hypoglycemia after insulin administration in the cat induced an increased secretion from the medulla. The glycogenolytic effect of adrenaline was thought to be an important mechanism for mobilizing blood sugar in hypoglycemia. HOUSSAY, LEWIS and MOLINELLI (1924) arrived at similar conclusions.

After intravenous injection of insulin sufficient to produce hypoglycemia in humans, EULER and LUFT (1952) demonstrated a large increase in the amount of adrenaline excreted in the urine whereas the amount of noradrenaline was little changed. Since the suprarenals are the most important source of adrenaline in the human body, it was concluded that the increased amount of adrenaline in the urine was due to an increased secretion from these glands. The administration of similar doses of insulin in patients with acromegaly was not followed by significant hypoglycemia and concomitantly the urinary adrenaline excretion remained essentially unaltered.

Conversely hyperglycemia decreases the catecholamine secretion from the suprarenal medulla and the adrenaline proportionally more than the noradrenaline secretion (DUNÉR 1953). In the same

[1] Aided by grants from Karolinska Institutet and from the Therese and Johan Andersson Memorial Foundation.

investigation there was a significant correlation between the blood sugar level and the amount of adrenaline secreted. There was also evidence that the blood glucose level influences the secretion from the suprarenal medulla by nervous pathways, via some form of glucose-sensitive receptors in the head, perhaps situated in the hypothalamus.

The present investigation is concerned with the influence of insulin hypoglycemia on the secretion of adrenaline and noradrenaline from the suprarenal of cat.

Material and Methods.

The cats used in the experiments were anesthetized with Nembutal 35 mg per kg body weight intraperitoneally. Blood from the left suprarenal vein was collected from a polyethylene tubing inserted into the corresponding lumbar vein. The arterial pressure of the cat was kept constant by means of a connection between the femoral artery and a reservoir filled with a warmed heparinized blood-Ringer solution (1/1) under a constant pressure. This was done in order to avoid influences from variation in the blood pressure on the secretion from the suprarenal medulla. The blood pressure was recorded from the other femoral artery. Arterial blood samples for blood sugar determinations (method of HAGEDORN, HALSTRÖM and JENSEN 1935) were collected from a brachial artery. Glucose solutions (10 %) and insulin were administered intravenously.

The suprarenal venous blood samples were centrifuged within 30 minutes after collecting. The serum was removed and stored in a refrigerator (— 30°C) and thawed immediately before testing. Adrenaline and noradrenaline were determined by assaying the suprarenal venous serum on cat's blood pressure and fowl's rectal caecum according to EULER (1949).

The experiments started with collecting control samples for blood sugar determination and control samples of the "resting" secretion of adrenaline and noradrenaline from the suprarenal. Insulin was then given and further samples for blood sugar determinations and samples of the suprarenal venous blood were collected at regular intervals. At the end of some experiments glucose solution was injected and further samples withdrawn.

Results.

The resting secretion of adrenaline in the 6 experiments ranged from 0.8 to 11 mμg/min./kg (mean: 6.2 mμg/min./kg) and for noradrenaline from 20 to 75 mμg/min./kg (mean: 37 mμg/min./kg). The per cent of adrenaline in the total resting secretion of adrenaline and noradrenaline ranged from 1 to 26 % (mean: 17 %).

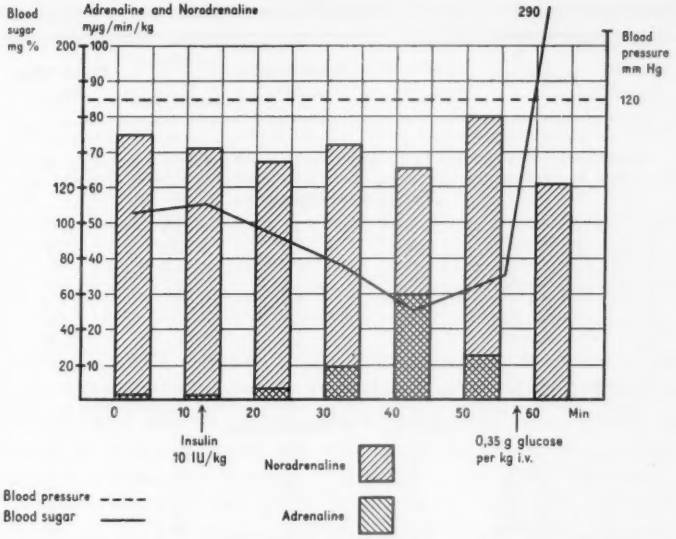

Fig. 1. Schematic diagram of experiment 1. Insulin, 10 IU per kg body weight injected i.v. after 12 min. and 0.35 g glucose per kg body weight infused 57 min. after the beginning of the experiment.

After intravenous administration of 10 IU insulin per kg body weight the greatest fall in the blood sugar level was usually noted after 35 minutes. The decrease of the blood sugar was accompanied by an increase up to tenfold or more of the adrenaline secretion from the suprarenal. The maximum secretion was reached at about the same time as the minimum blood sugar (see table 1). When the blood sugar level increased the adrenaline secretion decreased. Hyperglycemia due to the intravenous injection of glucose was in one experiment accompanied by the total disappearance of demonstrable adrenaline in the suprarenal venous serum (see fig. 1). The noradrenaline secretion also appeared to be influenced by the changes in the blood glucose level but to a much slighter and less consistent degree than that of adrenaline.

Discussion.

A differentiation of the secretion of adrenaline and noradrenaline from the suprarenal has been recently postulated. HÖKFELT

Table 1.

No. of sample	Time in min.	Adrenaline mμg/min./kg	Noradrenaline mμg/min./kg	Adrenaline % of Adr.+Nor.	Blood sugar mg%
Experiment 1. Cat 3.8 kg. 10 IU insulin per kg body weight injected i.v. after 12 min. and 0.35 g glucose per kg body weight infused 57 min. after the beginning of the experiment.					
1.....	0—5	0.8	75	1.1	105
2.....	10—15	0.4	71	0.6	110
3.....	20—25	3.0	67	4.3	95
4.....	30—35	9.1	72	11	75
5.....	40—45	30	65	32	50
6.....	50—55	13	80	14	70
7.....	60—65	—	61	—	290
Experiment 2. Cat 4.0 kg. 10 IU insulin per kg body weight injected i.v. after 10 min. and 0.25 g glucose per kg body weight infused 56 min. after the beginning of the experiment.					
1.....	0—5	10	29	26	105
2.....	15—20	16	35	31	100
3.....	30—35	48	32	60	80
4.....	40—45	120	44	73	60
5.....	50—55	71	42	63	65
6.....	60—65	8.0	27	23	230
Experiment 3. Cat 5.1 kg. 10 IU insulin per kg body weight injected i.v. after 10 min. and 0.25 g glucose per kg body weight infused 56 min. after the beginning of the experiment.					
1.....	0—5	7.1	22	24	110
2.....	5—10	5.3	20	21	105
3.....	30—35	7.5	24	24	90
4.....	40—45	12	25	32	70
5.....	50—55	17	30	36	70
6.....	60—65	3.1	25	11	270
Experiment 4. Cat 4.3 kg. 10 IU insulin per kg body weight injected i.v. after 10 min.					
1.....	0—5	4.2	20	17	90
2.....	15—20	4.0	15	21	95
3.....	30—35	9.3	27	26	85
4.....	40—45	30	22	58	60
5.....	50—55	32	25	56	60
6.....	60—65	18	19	49	75
Experiment 5. Cat 4.0 kg. 10 IU insulin per kg body weight injected i.v. after 10 min.					
1.....	0—5	4.8	54	8.2	120
2.....	15—20	7.1	50	12	110
3.....	30—35	12	62	16	70
4.....	40—45	50	70	42	55
5.....	50—55	57	61	48	55
6.....	60—65	13	50	21	85

No. of sample	Time in min.	Adrenaline mμg/min./kg	Noradrenaline mμg/min./kg	Adrenaline % of Adr. + Nor.	Blood sugar mg%
Experiment 6. Cat 4.1 kg. 10 IU insulin per kg body weight injected i.v. after 10 min.					
1.....	0—5	11	39	22	90
2.....	15—20	7.0	32	18	80
3.....	30—35	13	30	30	65
4.....	40—45	30	41	42	40
5.....	50—55	43	52	41	50
6.....	60—65	27	47	36	65
7.....	70—75	19	45	30	70

(1951) showed a selective depletion of adrenaline from the suprarenal of rat following the administration of insulin. BRÜCKE, KAINDL and MAYER (1952) found that hypothalamic stimulation gave rise to an increased per cent of adrenaline in the secretion from the suprarenal medulla. Supports for such a differentiated secretion has also been given in the earlier mentioned work of EULER and LUFT (1952) and EULER and FOLKOW (1953). Recently HÖKFELT and HILLARP (1953), using a histochemical method, have given evidence that adrenaline and noradrenaline are present in different cells in the suprarenal.

In the present investigation a marked increased secretion of adrenaline from the suprarenal medulla induced by insulin hypoglycemia was demonstrated by analysis of the suprarenal venous blood of cat; the noradrenaline secretion showed a slight tendency to increase. Under the given conditions, during periods of hypoglycemia, as earlier shown for hyperglycemia (DUNÉR 1953), there is an inverse proportion between the blood glucose level and the amount of adrenaline secreted from the suprarenal.

Thus the blood glucose level mainly influences the adrenaline secretion. The results give further support to the theory of a differentiated secretion of adrenaline and noradrenaline from the suprarenal medulla.

Summary.

The secretion of adrenaline and noradrenaline from the suprarenal was determined by the biological assay of suprarenal venous serum collected from nembutalized cats. Insulin hypoglycemia

was accompanied by a significant increase of the adrenaline secretion up to tenfold or more compared to the resting secretion. The results support the theory of a differentiated secretion of adrenaline and noradrenaline from the suprarenal, the blood glucose level chiefly influencing the adrenaline secretion.

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From Statens Serum Institut, Copenhagen.

On the Influence of Age on Plasma Protein Concentration, Blood Cell Volume, and Sedimentation Rate in the Ox.

By

POUL WEHMEYER.

Received 9 March 1954.

The purpose of the present study was to investigate changes in the composition of the blood of the ox during growth from birth to adulthood. JAMESON et al. (1942) found, that the relative γ globulin concentration was low in serum of the newborn, but it increased after intake of colostrum. HOWE (1921—1922) found an increase in albumin and globulin during growth. It was considered desirable to repeat such a study with improved technique and with investigation of the health of the animal.

Material and method: The present material comprised five healthy oxen, all red danish milkers. The blood composition of two males and three females was followed from the age of five days to twenty months with monthly examinations. In addition blood from each animal was examined three times on one day at the age of nine months and nine times in the course of one month at the age of four to five months. During the experimental period the animals were under veterinary observation and showed no signs of disease, including undulant fever or tuberculosis.[1]

The animals received colostrum during the first two days of life and whole milk for one month. In the summer season, they were on pasture; in the winter season they were stabled and received hay, grain, and water at 9 a. m. With only a few exceptions all blood samples were drawn at 7 a. m.

[1] I am greatly obliged to POUL HOLM, V. S., Statens Serum Institut for inspecting of the animals.

Serum protein and its albumin and globulin fractions were precipitated with saturated ammonium sulfate and determined gravimetrically according to the method of REYMANN (1924). Fibrinogen was determined from 2 ml citrated plasma after addition of 9 ml saline + 1 ml 0.18 calcium chloride according to the procedure described by GRAM (1921). Moreover red cell volume in per cent of total blood volume and sedimentation rate in mm per 24 hours were determined on each blood sample. For comparison blood samples of one animal were analysed by electrophoresis in a Tiselius apparatus modified according to SVENSSON (1946). The serum pH was adjusted to 7.7 and the ionic strength to 0.1. For each serum sample the relative concentration of albumin, of α, β, and γ-globulin was determined on the *positive* side of the electrophoresis cell. From their relative concentrations the protein fractions were calculated.

Table 1.

Survey of the Accidental Variation in the Concentration of Plasma Proteins and Cell Volume in 5 Calves.

	Standard deviation of the method	Standard deviation	
		in one day	in one month
Protein	0.03	0.17	0.20
Albumin	0.05	0.07	0.12
Globulin	0.06	0.12	0.23
Fibrinogen	0.04	0.03	0.03
Cell volume	1.3	1.5	1.6

Table 2.

Serum Protein Concentration and the Absolute Concentrations of Albumin, α, β and γ-Globulin determined by Electrophoresis calculated on the Positive Side of the Electrophoresis Cell for Ox No. 1444.

Age	Grams protein per 100 ml serum	Positive side Absolute concentration of			
		Albumin	Globulin		
			α	β	γ
5 days	4.63	2.98	0.56	0.59	0.50
1 month	5.46	3.14	0.80	0.65	0.88
3 months	5.78	3.44	0.64	0.58	1.12
4 »	6.22	3.62	0.63	0.55	1.42
8 »	6.34	3.94	0.39	0.53	1.49
10 »	6.80	4.15	0.56	0.49	1.60
14 »	6.22	3.91	0.45	0.53	1.32
19 »	7.10	3.96	0.68	0.77	1.69

Results.

Examination of the blood samples taken in the course of one day showed that the concentration of serum protein and globulin varied in the course of the day (Table 1, column 2). The examinations performed in the course of one month showed an accidental variation in the concentration of albumin as well. (Table 1, column 3.) Some of the changes in cell volume occurred equally in all animals and must be considered as systematic variation. They could not be correlated to other changes in the blood composition.

Investigation into the influence of the age of the animal upon the composition of the blood gave the following results:

At the age of five days the five calves showed an average albumin concentration of 2.57 per cent (Fig. 1). During the following two months it rose to the level previously found for adult cows ($3.04 + 0.09$ per cent ($f = 7$) WEHMEYER 1954). The average serum globulin and protein concentrations were respectively 2.29 and 4.86 per cent at the age of five days. In the course of the following nineteen months the serum globulin (and protein) concentrations increased to the average values found for adult cows; 4.32 ± 0.17 per cent for globulin ($f = 7$), and 7.41 ± 0.10 per cent for total protein ($f = 37$).

Electrophoresis of the blood samples from calf 1444 showed that the serum albumin and γ globulin concentrations increased with the age of the animal (Table 2). The albumin concentration reached in about 4 months the level of corresponding values found for adult cows, and the γ globulin concentration rose to the corresponding level in about 20 months. 3.85 ± 0.11 per cent for albumin and 2.05 ± 0.09 for γ globulin ($f = 9$). (WEHMEYER 1954.)

The average fibrinogen concentration showed only slight variation throughout the experimental period. It did not vary with changes in the serum protein. Individual differences between different animals persisted throughout the experimental period of 19 months (Figs. 1 and 2). The cell volume was about 35 per cent at five days; the sedimentation rate about 8 mm. The rise in the serum protein concentration was in the first half year accompanied by a fall in the cell volume from 35 to 28 per cent and by an increase in the sedimentation rate from 8 to 18 mm per 24 hours. After twelve months the cell volume increased

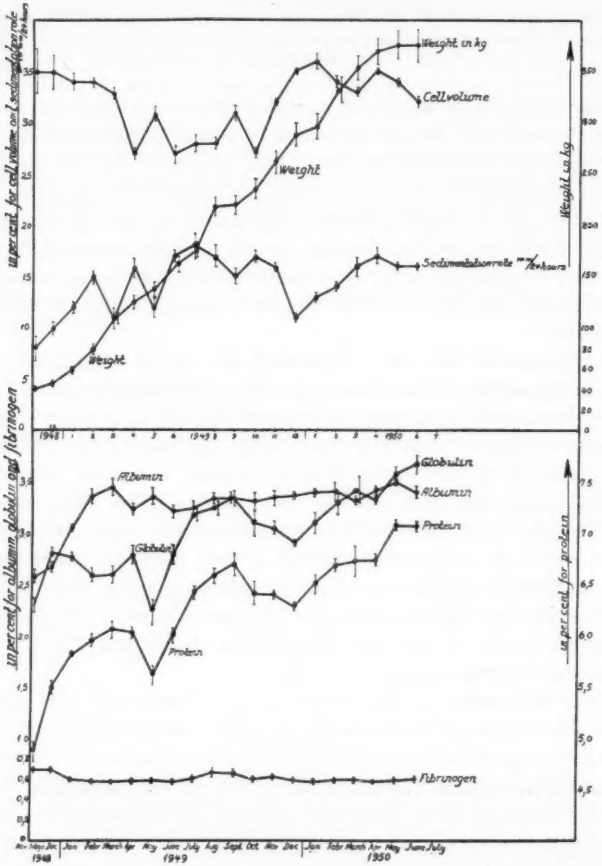

Fig. 1. *Lower half of the figure:* Concentration of serum protein (right ordinate); of albumin and globulin in g per 100 ml serum and of fibrinogen in g per 100 ml plasma (left ordinate).

Upper half of the figure: Cell volume in per cent of total blood volume, sedimentation rate in mm per 24 hours (left ordinate) and weight in kg (right ordinate). Abscissa: time in months. Each point represents a mean value of five calves. The mean error (4 degrees of freedom) is indicated by the vertical line at each point.

slowly again to the values found for adult cows: 34.8 ± 0.8 per cent ($f = 37$). No dependence between fibrinogen concentration and sedimentation rate was demonstrated. The average weight of the animals in this group increased gradually through the greater part of the experimental period.

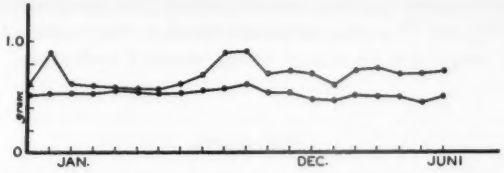

Fig. 2. Fibrinogen concentration determined once a month for calves 1442 and 1445, from age of 5 days. Note the individual difference. Abscissa: Time in months. Ordinate: The fibrinogen concentration in grams per 100 ml plasma.

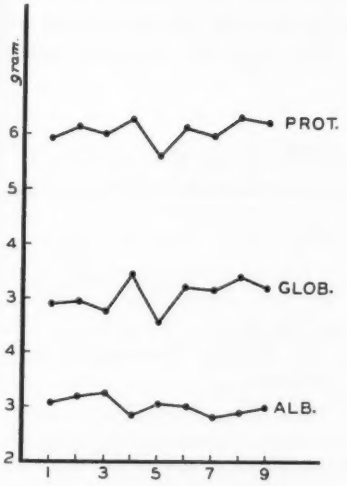

Fig. 3a.

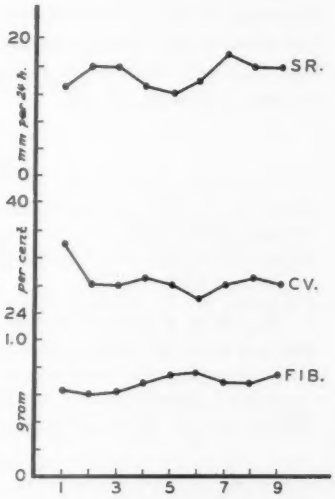

Fig. 3b.

Fig. 3a. Concentration of serum protein, albumin, and globulin determined nine times in the course of one month on calf 1441. Abscissa: Number of experiments. Ordinate: The concentration of serum protein, albumin, and globulin in grams per 100 ml serum.

Fig. 3b. Fibrinogen concentration (FIB), cell volume (CV), and sedimentation rate (SR) determined nine times in the course of one month on calf 1441. Abscissa: Number of experiments. Ordinate: The concentration of fibrinogen (in grams per 100 ml plasma), cell volume (in per cent) and sedimentation rate (in mm per 24 hours).

The variations in the composition of blood in a single day, and within one month, were as follows:

The globulin varied more than the albumin concentration (Table 1) and the two varied independently of each other. The

variation in serum protein concentration was independent of cell volume (Fig. 3). The changes in the blood in the course of one day or month could not be related to the state of hydration.

Summary.

The influence of age upon the composition of the blood was studied in five oxen. In addition, daily or monthly variations were studied.

Within three months the albumin concentration and within nineteen months the globulin (γ globulin) concentration reached the adult level. The fibrinogen concentration did not depend on age. The cell volume decreased after birth and increased again at twelve months of age.

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From the Zoophysiological Laboratory, Department of Biological Isotope Research, and the Institute for Theoretical Physics, University of Copenhagen.[1]

On the Age of Human White Cells in Peripheral Blood.

By

J. OTTESEN.

Received 10 March 1954.

The white cells are usually assumed to spend a rather short time in the blood stream. In a recent review, YOFFEY (1950) states that the lymphocyte population in the blood of the rabbit is replaced 5 times in the course of 24 hours. Similar values are reported for the rat, cat, and dog. VAN DYKE and HUFF (1951) found that the average time the granulocyte spends in the blood of the rat is 23 minutes, while the corresponding time for the lymphocyte is 170 minutes. These figures are in agreement with calculations on the basis of the mitotic counts of KINDRED (1942). It was emphasized by YOFFEY (1950) that, with regard to the lymphocyte, the above results afford no evidence as to the time the cells live in the organism. Obviously, a similar statement holds for the granulocytes. MILLS (1949) estimated the life span of rat granulocytes as about 2 to 3 weeks in nutritional experiments in which he followed the change in the phagocytic activity of these cells. By studying the fate of leukocytes labelled by incorporation of ^{32}P in their desoxyribonucleic acid, OTTESEN (1948) found that most of the labelled cells had disappeared from the blood of a hen within 8 days after the injection of ^{32}P. KLINE and CLIFTON (1952) labelled human leukocytes in a similar way and found that the bulk of the labelled cells remained 4 days in the bone marrow before entering the blood stream, and that

[1] The investigations were performed on patients hospitalized in the Department of Internal Medicine at the Finsen Memorial Hospital, Copenhagen, Dr. A. HECHT JOHANSEN, Chief.

most of the labelled cells had disappeared from the blood about 13 days after the administration of the isotope.

In the present work, human white cells are labelled by incorporation of ^{32}P in their desoxyribonucleic acid. On the basis of radioactivity measurements, determinations are made of the time which the circulating white cells have spent in the organism. This time is denoted the age of the cells. Lymphocytes and granulocytes are measured separately. While the majority of the lymphocytes are found to have a mean age of about 100 to 200 days, a minority have a mean age of about 3 days. The granulocytes are found to have a mean age of about 9 days.

Basic Considerations.

When cells are formed by mitosis, we can assume that the new cells contain almost exclusively new DNA which was synthesized during a part of the interphase. In the mature cells, the turnover of DNA probably is negligible. This will be discussed in more detail below. During synthesis, phosphorus from an immediate precursor, an intracellular phosphorus compound, is incorporated in DNA. On the basis of the specific activity of the precursor and that of the DNA phosphorus of the white cells in the blood stream, it is possible to calculate the age of the cells, as described later.

Experiments on the lymphoid organs of the rat (ANDREASEN and OTTESEN 1952) suggest that the specific activity of the immediate precursor of the DNA phosphorus is nearly equal to that of the intracellular inorganic phosphate. Within a few hours, the specific activity of inorganic phosphate in the cells becomes equal to that of the plasma inorganic phosphate (ANDREASEN and OTTESEN, 1945). Similar results were obtained by HULL and KIRK (1950). These authors assume that, in tissue cultures of chicken heart fibroblasts, the immediate precursor has the same specific activity as the inorganic phosphate of the culture medium. Experiments on the life span of hen red cells (OTTESEN 1954) suggest that the specific activity of plasma inorganic phosphate represents a satisfactory approximation to that of the immediate precursor of the DNA phosphorus in the red cells. By this approximation, the error caused in the average time for the appearance of the cells in the blood stream was less than one day. Thus, in the present experiments, the specific activity of the plasma

inorganic phosphate may be assumed to represent a satisfactory approximation to the specific activity of the precursor of the DNA phosphorus. Technical reasons, however, make the measurement of the specific activity of plasma inorganic phosphate rather inconvenient.

The inorganic phosphate excreted in the urine has the same specific activity as the plasma inorganic phosphate, although at first there is a small difference between the curves, as demonstrated by HANDLER and COHN (1951) in experiments on dogs. This difference disappeared within 25 minutes following intravenous injection of ^{32}P, and was explained on the basis of a lag between the time at which inorganic phosphate is filtered by the glomerulus and the time at which it reaches the bladder.

As described later, the calculations of the age of the cells is based on the average value of the specific activity of the precursor during each 24 hour time interval of the experiment. These values are conveniently obtained from urine samples collected during each time interval. When the ^{32}P is injected at a suitable time of the day, viz. about noon, the diurnal variation in the quantity of phosphate excreted per hour can be neglected (cf. FISKE 1921). It is then reasonable to assume that the specific activity of the phosphate in a urine sample collected in the course of a 24 hour period represents a good approximation to the average value of the specific activity of the plasma phosphate and, therefore, that of the precursor during the same period.

Experimental Method.

The experiments were performed on patients who were free of diseases involving the white blood cells and who had normal total and differential blood counts.

Patient B., an 88-year-old woman suffering from coronary thrombosis, was treated with heparin and cumarol during the experiment.

Patient H., a 44-year-old woman with a gastric ulcer, received no special medical treatment, but was examined by means of X-rays twice during the experiment.

The radiophosphorus, 200 μC, was injected intravenously.

Blood samples were taken from the cubital vein. Nine ml of blood was drawn into a glass syringe containing 9 ml of an ice-cold solution of 0.7 per cent sodium oxalate and 1.9 per cent sodium citrate. The blood and the saline solution were mixed thoroughly and as quickly as possible, immediately poured into a test tube placed in ice water,

and transported to the laboratory. Clumping of the leukocytes could be almost completely avoided by cooling the blood cells at once and keeping them at between 0 and 5°C during the separation of the white cells.

The separation of lymphocytes and granulocytes was carried out by making use of their differences in both sedimentation rate and specific gravity. At first, the lymphocytes were removed from the bulk of the

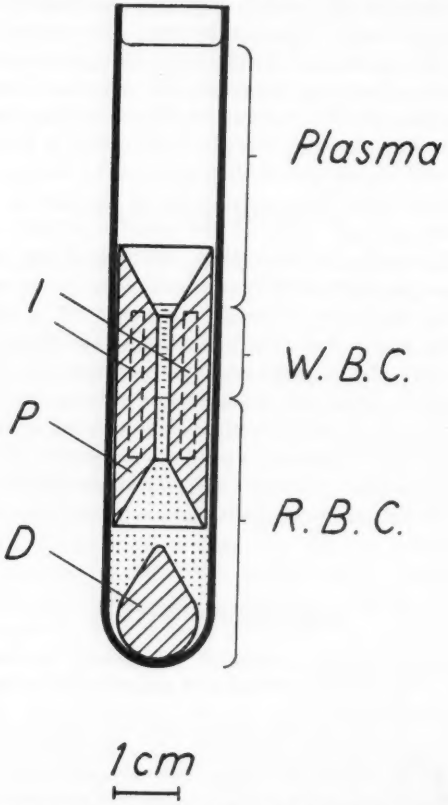

Fig. 1. Centrifuge tube with perspex cylinder P, for separating red and white blood cells. The specific gravity of the perspex cylinder is adjusted to 1.052 by means of the internal cavities, I. Before centrifugation, when the perspex cylinder has sunk to the bottom of the blood, the space between the cylinder and the bottom of the centrifuge tube is filled by the drop-shaped perspex body, D. During the centrifugation almost all the red cells go through the capillary because of the funnel-shaped upper end of the perspex cylinder. Some of the white cells which are drawn down by the red cells, are allowed to swim up in the buffy coat through the funnel-shaped lower end of the perspex cylinder.

granulocytes and erythrocytes by centrifugation in an ordinary centrifuge tube in which was placed a cylindrical perspex body with a capillary tube in its axis (Fig. 1). The buffy coat was located in this capillary tube after centrifugation. By means of internal cavities, the specific gravity of the perspex cylinder was adjusted to 1.052, so that it would sink to the bottom of the blood saline mixture but float on the layer of red cells when these were thrown down in the process of centrifugation. Thus, the location of the surface between the red and white cell layers could be adjusted to the middle of the capillary. After centrifuging the material for one hour at 2,300 rpm, the white cell column and the uppermost red cells were sucked off by means of a capillary tube. In this way, about half of the lymphocytes but only few granulocytes were removed. Subsequently, the red cells were resuspended in the plasma saline mixture and the material was centrifuged once more. It was found sufficient to repeat the procedure twice.

The pooled fractions of white cells obtained in this way were further purified by centrifugation in a gradient tube, utilizing the differences in density. A narrow centrifuge tube (4 mm inner diameter, 110 mm length, and funnel-shaped at the open end) was filled to the middle with a solution of 300 mg glycogen in one ml of 0.45 per cent sodium chloride and 1.9 per cent sodium citrate. Above this, a mixture similar in salt content but without glycogen was carefully introduced, while about 1.5 cm at the upper end of the tube was left free to receive the white cell mixture. A density gradient was produced by stirring with a copper wire (see HOLTER et al., 1953), the tube was cooled in ice, and after the lapse of an hour it was ready for use. The white cell mixture was carefully introduced into the top of the gradient, and the tube was centrifuged for 1.5 hours at 2,300 rpm. The centrifuge was started and stopped slowly to avoid disturbing the gradient. After centrifugation, the following layers were seen in the gradient tube, from top to bottom: a thick white layer, mainly thrombocytes and a few lymphocytes; a thin white layer, mainly lymphocytes; a thin red layer containing some lymphocytes, some granulocytes and some erythrocytes, and, at the bottom of the tube a red bulk of erythrocytes. These layers were sucked off separately by means of a capillary tube. The pooled white layers comprised the lymphocyte fraction.

The red layers in the gradient tube were mixed with the erythrocytes under the perspex body in the centrifuge tube, and these pooled cells were hemolyzed by adding about 8 ml of a solution of 0.16 per cent saponin in 0.9 per cent sodium chloride. The tubes were cooled in ice water during the hemolysis which usually was completed within 5 minutes. The granulocyte fraction of the white cells was obtained after centrifugation and washing by resuspension in 1 to 2 ml of the plasma saline mixture with subsequent recentrifugation.

Smears were made of the two white cell fractions, preferably while the cells were resuspended in the plasma-saline mixture. The lymphocyte fraction usually contained 94 to 98 per cent lymphocytes, some monocytes, and a few granulocytes. In the granulocyte fraction the polymorphonuclears and monocytes together represented 91 to 96 per

cent of the white cells. An admixture of as much as 10 per cent of red cells is permissible since it is only the DNA phosphorus which is under investigation.

After separation, the two white cell fractions were transferred to conical, pointed-bottomed pyrex centrifuge tubes. They were centrifuged, and then resuspended in alcohol.

The desoxyribonucleic acid phosphorus of the white cells was prepared by a simplification of the method of SCHMIDT and THANNHAUSER (1946). The phosphatides were extracted by suspending the cells in a boiling alcohol-ether mixture (2 parts alcohol to 1 part ether), and centrifuging. This extraction was repeated once and followed by an extraction with ether at room temperature, performed in the same way. The cells were dried at 37°C, dissolved in 0.5 ml 1 N sodium hydroxide, and maintained at 37°C until the next day. Then protein and DNA phosphorus were precipitated by addition of 0.2 ml 3 N hydrochloric acid and 0.25 ml 10 per cent trichloroacetic acid. The material was centrifuged and washed once by suspension in 3 per cent trichloroacetic acid, followed by another centrifugation.

The DNA phosphorus from blood samples obtained within two days after the injection of ^{32}P was further purified for at the time these samples were taken the inorganic P of the body pool had a rather high specific activity. The precipitate was dissolved in 0.5 ml 1 N sodium hydroxide and precipitated as described above. This purification was repeated once.

For wet decomposition of the precipitate, 70 μl of a mixture of 3 parts conc. nitric acid and 1 part conc. sulphuric acid were added. The centrifuge tubes were now placed in a beaker with sulphuric acid on the bottom in a layer about 5 mm thick (*e. g.* six tubes in a beaker of 7 cm diameter and 9 cm high). This sulphuric acid bath was kept at 130° to 150°C during the decomposition. When the mixture became dark, a small drop of conc. nitric acid was added. Finally, the temperature was raised to 220° to 250°C and, if the mixture still was clear after cooling, decomposition was considered complete. Then 1 ml of water was added and the pyrophosphate formed during the decomposition was hydrolysed by immersing the centrifuge tubes in a boiling water bath for 20 minutes.

The phosphorus was precipitated as magnesium ammonium phosphate, and the radioactivity was measured in an apparatus as described by AMBROSEN, MADSEN, OTTESEN and ZERAHN (1945). The quantity of ^{31}P in the samples was determined colorimetrically according to KJERULF-JENSEN (1942).

The urine was collected during each 24 hour period and the samples were preserved by addition of hydrochloric acid. The specific activity of the urine inorganic phosphate was determined as described above.

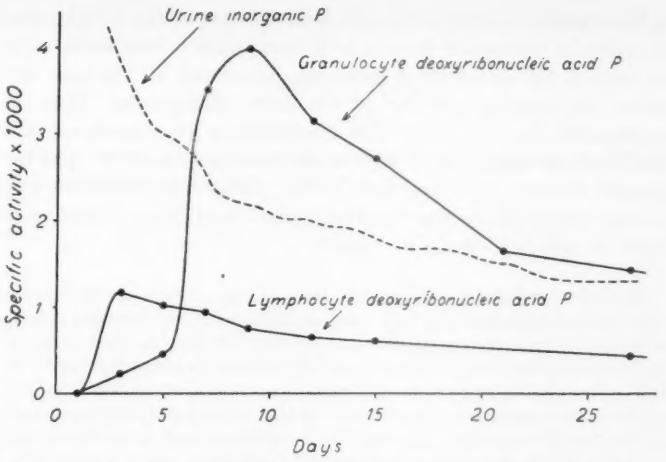

Fig. 2. Specific activity (per cent of injected ^{32}P per mg P) of urine inorganic phosphate, granulocyte DNA phosphorus, and lymphocyte DNA phosphorus, plotted against time. The mean specific activity of urine inorganic phosphate during the first day of the experiment was found to be 17.4×10^{-3}, and that during the second day 5.71×10^{-3}.

Calculation of the Age of the Cells in the Blood Stream.

Time-course of the specific activities. In Fig. 2, the results of investigations on patient B. are presented. The specific activity[1] of inorganic phosphorus of the urine decreases rapidly during the first days after the injection, and slowly later on. The curve is somewhat irregular, possibly because of variations in the amount of phosphorus consumed daily with the food.

The specific activity of the desoxyribonucleic acid phosphorus both of the granulocytes and the lymphocytes is zero one day after the injection, indicating the stability of desoxyribonucleic acid P in the white blood cells. In the period from 1 to 5 days after the injection, the specific activity of the DNA phosphorus of the granulocytes increases slowly, while from 5 to 7 days the increase is very rapid. A maximum is reached well above the urine curve. The steep rise of the curve indicates that, about 6 days after the injection, the bulk of labelled granulocytes is introduced into the circulation from the bone marrow.

[1] In the present paper, specific activity denotes per cent of injected ^{32}P found per mg phosphorus in sample.

The specific activity of the DNA phosphorus of the lymphocytes increases in the period from 1 to 3 days, when a maximum value is reached. Subsequently it decreases, remaining all the time well below the specific activity of the urine phosphorus. Thus, an appreciable number of labelled lymphocytes are introduced into the blood stream about 2 days after the injection of ^{32}P. The low specific activity of lymphocyte DNA phosphorus indicates that a large group of the lymphocytes in the blood stream was formed prior to the injection (cf. below).

Method of calculation. While the time of appearance of the labelled cells in the blood stream can be estimated immediately from the specific activity curve, special calculations are needed for the determination of the age of the circulating cells at later dates. As the precursor of the DNA phosphorus retains an appreciable activity during the later part of the experiment, the cells formed at that time contribute essentially to the specific activity of the DNA phosphorus, and from the specific activity curve we cannot immediately estimate when the strongly radioactive cells from the initial part of the experiment have disappeared from the blood stream. In the following, a method is outlined which allows the calculation of the fraction of the white cell DNA phosphorus in the blood — formed during a short time interval of unit length at the start of the experiment — and which is still present in the blood stream at the time t. This fraction is denoted by $\varphi(t)$.

The time of the experiment is divided into convenient intervals of, say, 24 hours each. The average specific activity of the precursor is designated $f(^1/_2)$ during the first time interval, $f(1^1/_2)$ during the second, etc. The specific activity of the white cell DNA phosphorus in the blood stream after the lapse of one day is g(1); after the lapse of two days it is g(2), etc. The fraction of the white cell DNA phosphorus in the blood stream which is produced and poured out into the blood stream in the course of the first day, *i. e.* the fraction which is renewed (cf. HEVESY, 1948 a) works out to be

$$\varphi(^1/_2) = \frac{g(1)}{f(^1/_2)}. \tag{1}$$

Thus, $\varphi(^1/_2)$ indicates the fraction of the blood DNA phosphorus which has an age from zero to one day, *i. e.* an average age of one half day. As we assume a steady state during the experiment, each day the same number of white cells is produced. These cells are later poured out into the blood stream in the course of several days. During the second day, the number of white cells produced and transferred to the blood stream is the same as during the first day. This time, however, the average specific activity is $f(1^1/_2)$. Thus, the contribution to the specific activity in the blood stream is $f(1^1/_2)\varphi(^1/_2)$ and the part of the specific activity which originates from the first day's production is now $g(2) - f(1^1/_2)\varphi(^1/_2)$. The fraction of the first-day-production of white cells in the blood

stream which now has the age from one to two days (*i. e.* the average age $1^1/_2$ day) works out to be:

$$\varphi(1^1/_2) = \frac{g(2) - f(1^1/_2)\varphi(^1/_2)}{f(^1/_2)} . \tag{2}$$

Similarly, the fraction of the white cell DNA in the blood stream which has the average age $2^1/_2$ days is found to be:

$$\varphi(2^1/_2) = \frac{g(3) - f(1^1/_2)\varphi(1^1/_2) - f(2^1/_2)\varphi(^1/_2)}{f(^1/_2)} , \text{ etc.} \tag{3}$$

In this way we obtain an equation for every time interval. The equations are easily solved by substitution, and the calculations are conveniently carried out by means of an electrical calculating machine when the number of equations is about 20 or less. In every interval the $\varphi(t)$'s obtained in this way represent the specific activity of the DNA phosphorus that would have been measured at some time in the interval if the specific activity of the precursor had had the value one during the first interval and the value zero during the remaining time of the experiment (compare the specific activity curves of Fig. 2 and the $\varphi(t)$ curves for patient B. of Fig. 3 and Fig. 4). The method outlined above is described in detail elsewhere (Ottesen 1954), and there an estimation of the error is given also. It is shown that the $\varphi(t)$'s obtained by solution of the equations (1), (2), (3), etc. in fact represent an approximate solution of the integral equation:

$$g(t) = \int_0^t f(\theta)\varphi(t - \theta)d\theta \tag{4}$$

which is similar to the integral equation used by Shemin and Rittenberg (1946) in experiments on red cells, and by Branson (1948, 1952) in experiments where a random destruction of the labelled compound took place.

As mentioned below, we can assume that the DNA phosphorus is incorporated in the cell nuclei prior to mitosis and remains there throughout their lifetime. Thus, the labelling of the DNA phosphorus represents a labelling of the cells, and $\varphi(t)$ indicates that fraction of the white cells in the blood stream which has the age t and was formed during a time interval of unit length. The fraction of the cells in the blood which has an age less than t_1 is

$$\int_0^{t_1} \varphi(t)dt .$$

As all cells have an age between zero and infinity, we have

$$\int_0^{\infty} \varphi(t)dt = 1 . \tag{5}$$

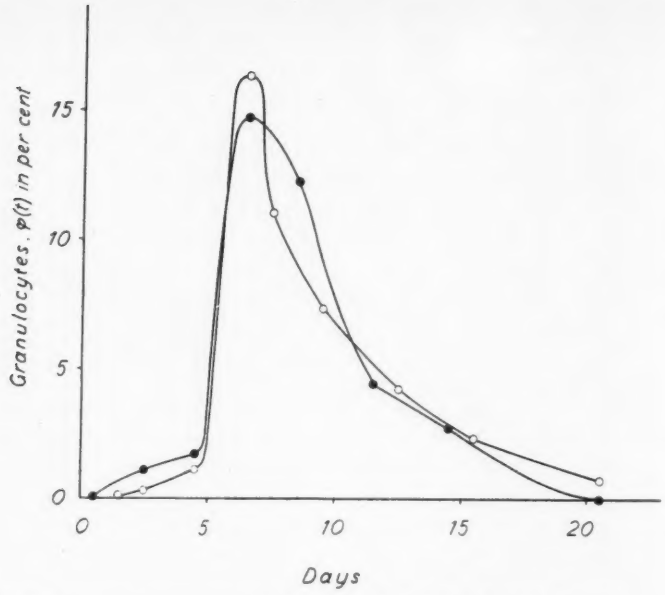

Fig. 3. Percentage of granulocytes, formed during the first day of the experiment and present in the blood stream at the time indicated on the X-axis. Patient H., open circles, —o—o—, patient B., solid circles, —●—●—.

The mean age of the white cells in the blood stream is

$$T = \frac{\int_0^\infty t\varphi(t)dt}{\int_0^\infty \varphi(t)dt}. \tag{6}$$

The integrals are evaluated from the $\varphi(t)$ figures by numerical methods.

Results.

The granulocytes. Fig. 3 shows the curves calculated on the basis of the specific activity data for patient B. and patient H. The curves are essentially similar. The negligible value for the first day is followed by a slow increase until about 6 days, when a sharp rise sets in. From the maximum value at 6.5 days the curves descend slowly. The curve for patient B. reaches zero after

about 20 days. Unfortunately, the experiment on patient H. was discontinued at 21 days after the injection since the patient was released from the hospital at this time. Continuation of the experiment might have yielded zero values.

From the curve for patient B. the integral $\int_0^\infty \varphi(t)dt$ is found to be 0.89 and the curve for patient H. has the integral 0.87. The deviation from the theoretical value one presumably can be explained by systematic experimental errors in the specific activities of the DNA phosphorus samples.

Unlike the red blood cells, which spend most of their lifetime in the blood stream, the white cells are in the blood stream only en route to the organs. The results of cross-circulation experiments by LAWRENCE et al. (1945), are in favour of the idea that the longest time the granulocytes spend in the blood is not much longer than the mean time, and that only a negligible number are present in the blood stream during more than one day. Thus, the bulk of the white cells introduced into the blood stream during the 5th day should leave the blood within a day or less and be replaced by other myeloid white cells entering the blood from the marrow.

The low specific activity of the DNA phosphorus of the granulocytes in the blood one day after the injection of ^{32}P indicates the stability of the labelling of the cells in the blood and in the bone marrow within a day prior to entering the blood. If we assume that the ^{32}P is incorporated into white cell DNA phosphorus during the interphase (HOWARD and PELC, 1951), then the labelled first-day cells stay in the marrow for some days, presumably while a maturation process takes place, and many of them enter the blood on the fifth day and then disappear rapidly. During the following days, more of the labelled first-day cells enter the blood stream and disappear again. From about 20 days after the start of the experiment, no more of the labelled first-day cells enter the blood stream, presumably because the marrow store of these cells is now exhausted. Thus, a great fraction of labelled cells stay in the marrow for several days. During this time, the turnover of the labelled DNA phosphorus present in the cells is assumed to be negligible. This assumption is supported, on the one hand, by the observation that the turnover of DNA phosphorus in organs with few mitoses is minute (OSGOOD et al. 1951, HEVESY 1948 b, HEVESY and OTTESEN 1943) and, on the other

hand, by radioautographic results obtained by HOWARD and PELC (1951) and by TAYLOR (1953) which demonstrate the incorporation of ^{32}P into DNA phosphorus shortly prior to mitosis. Furthermore, HENSTELL, FREEDMAN and GINSBURG (1952) suppose the DNA metabolism to be absent in mature cells where the depolymerisation of DNA is inhibited.

A possible explanation of the slow rise of the calculated $\varphi(t)$ curve from 1 to 5 days is that a small percentage of the myeloid cells enter the blood within a shorter time after mitosis than does the main bulk of myeloid cells. For example, the so-called juvenile cells, as defined by the Schilling haemogram, might spend a shorter time in the marrow than do the segmented polymorphonuclears.

The maximum of the curve at about 7 days is followed by a rather slow decline. As mentioned above, this fall may be caused by a slow release of cells labelled during the first day of the experiment and located subsequently in the marrow. The experimental results, however, do not exclude the possibility that some of the strongly radioactive cells which were present in the blood stream at the maximum, and then went into the organs, return to the blood later. The experimental results give only the age of the cells in the blood stream. The mean age of the cells as defined above (Eq. 6) works out to be 8.7 days for patient B. and 9.4 days for patient H.

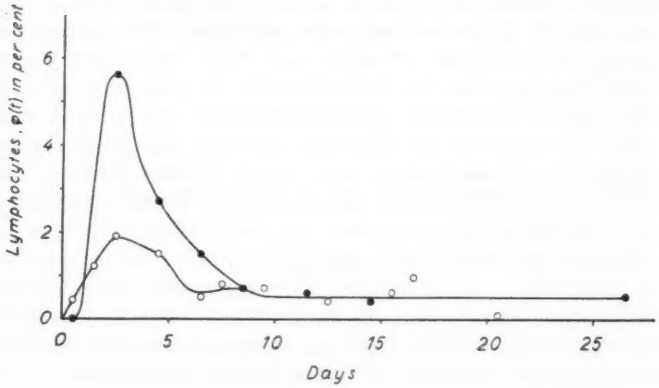

Fig. 4. Percentage of lymphocytes, formed during the first day of the experiment and present in the blood stream at the time indicated on the X-axis. Patient H., open circles, —o—o—, patient B., solid circles, —●—●—.

The lymphocytes. The curves for the lymphocytes calculated for patient B. and patient H. are seen in Fig. 4. Both curves show the introduction of a number of labelled lymphocytes into the blood stream at about 2 days, and a maximum number at 2.5 days. At about 10 to 14 days both curves have $\varphi(t)$ values of about 0.005.

For patient B., the rising and declining part of the curve, from zero to 11 days, has $\int_0^{11} \varphi(t)dt = 0.22$. If we should have $\int_0^{\infty} \varphi(t)dt = 1$ (Eq. 5), the following part of the curve should have $\int_{11}^{\infty} \varphi(t)dt = 0.78$. It would, for example, either continue horizontally to about 166 days, as $\varphi(t)$ is about 0.005, or follow an exponential curve to zero during a still longer time. The curve indicates that 22 per cent of the DNA phosphorus in the lymphocytes of the blood are renewed within 11 days, while the remaining 78 per cent is renewed at a much slower rate. The mean age of the short-living lymphocytes is roughly estimated to be about 4 days, and that of the long-living lymphocytes is about 100 to 170 days.

The $\varphi(t)$ curve for the lymphocytes of patient H. is essentially identical to the curve of patient B. For patient H., however, the part of the curve, from zero to 11 days, corresponding to short-living lymphocytes, has $\int_0^{11} \varphi(t)dt = 0.11$, and the mean age is estimated to be about 3 days. The mean age of the long-living lymphocytes is roughly estimated to be 100 to 180 days. The time course of the specific activity of the lymphocyte DNA phosphorus was not as regular as that for patient B. The irregularities are so small, however, that they do not invalidate the principal conclusion of the present experiments, namely, that the main part of the lymphocytes has a very long life span. If the irregularities were correlated with some disturbance of the steady state, the error in the calculated $\varphi(t)$ curve would be diminished as a result of the rapid decrease in the specific activity of the precursor.

Similar curves could be obtained if the DNA phosphorus were an intermediate product in a sequence of reversible reactions (cf. BARNUM and HUSEBY 1950). Then the DNA phosphorus might be formed simultaneously from the preceding product, having the same specific activity as urine inorganic phosphate, and from the following product which might represent a large

phosphorus reservoir. As yet, however, no phosphorus compound is known to occur in the cells in quantities similar to, or greater than, the DNA phosphorus and having a renewal rate slower than that of the DNA phosphorus. Hence, this interpretation of the curves must be rejected.

Without calculating the $\varphi(t)$ curve, similar conclusions could be drawn from the specific activity curves of Fig. 2; in this case, however, the relative amounts of the two fractions of DNA phosphorus could not be evaluated. The maximum of the specific activity of lymphocyte DNA phosphorus at three days is well below that of the urine curve, and the declining part of the lymphocyte curve continued to the end of the experiment without intersecting the urine curve. Such a curve is obtained when a fraction of the DNA phosphorus is renewed rather rapidly and the specific activity of this fraction is reduced by dilution with a greater fraction of "old", slowly renewed, nearly inactive DNA phosphorus. If we were able to separate the two fractions of DNA phosphorus, we would find that the specific activity of the rapidly renewed fraction rises above that of the precursor, and decreases while still remaining above that of the precursor. Since the two fractions are not separated, as a result of the dilution with the slowly renewed fraction of the DNA phosphorus, the specific activity is reduced so much that both the maximum and the following part of the curve remain below the values shown in the precursor curve.

Such a "dilution" might be the consequence of a reutilization of the phosphorus from old lymphocytes disintegrated in the lymphoid organs. Evidence against this hypothesis is obtained, however, from experiments on rats. A few preliminary experiments have shown that the lymphocytes of old rats contain a fraction of DNA phosphorus which also shows a low turnover rate, but this fraction is smaller than that found in humans (ANDREASEN and OTTESEN, 1952). If this fraction had emerged from incorporation of "old" inactive phosphorus in the new lymphocytes prior to mitosis, then the number of newly formed cells calculated from the mitotic figures would be greater than that calculated from the ^{32}P content of the DNA phosphorus of the lymphocytes. The rate of renewal of the DNA phosphorus has been determined by ANDREASEN and OTTESEN (1945), while the percentage of cells in mitosis is known from the work of ANDREASEN and CHRISTENSEN (1949). It may be noted that in both investiga-

tions animals from the same colony were used. In the calculation it has been assumed that a cell goes through the mitotic phases in the course of 21 minutes, as suggested by VAN DYKE and HUFF (1951), and within this time is divided into two daughter cells, each containing the normal quantity of DNA phosphorus. In this way, the ratio between the percentage of new-formed DNA phosphorus and the percentage of new-formed cells has been found to be 2 to 3. The fact that the quantity of new DNA phosphorus is greater than that of new cells may possibly be explained by a turnover of DNA phosphorus during the period when the ^{32}P is incorporated prior to mitosis. Simultaneously with the formation of new DNA phosphorus, "old" molecules are disintegrated (EULER and HEVESY 1942, 1944; AHLSTRÖM, EULER and HEVESY 1944).

As the percentage of new cells is smaller than that of new DNA phosphorus, we can assume that the new cells contain "new" DNA phosphorus only. Accepting this assumption, it can be inferred that the DNA phosphorus rapidly renewed in the blood stream corresponds to a rapidly renewed population of lymphocytes, while the DNA phosphorus which was not renewed to a significant extent during the experiment corresponds to a number of lymphocytes having a long life span. Furthermore, as circulating lymphocytes are diploid, and tetraploid cells are not found (PETRAKIS 1953), then the $\varphi(t)$ curve gives evidence as to the lymphocyte fraction in the blood stream which was formed during the first day of the experiment and is present in the blood stream at later dates. Thus, about 80 per cent of the lymphocytes present in the circulation must have been formed an appreciable time prior to the start of the experiment.

In the above estimation of the long life span of the main part of the lymphocytes it is assumed that the turnover of the DNA phosphorus is completely absent in the mature lymphocytes and that ^{32}P is incorporated only during mitosis. If a minute turnover should take place in the old cells, the life span would be longer than the estimated values of about 100 to 180 days. If the turnover of the DNA in the old lymphocytes in the lymph glands is as low as that found in mature hen erythrocytes in the blood stream (OTTESEN 1954), then the low $\varphi(t)$ values during the later part of the experiment may possibly be explained in the following way: The majority of those lymphocytes that are formed during the first day of the experiment, and are not released within 10 days, remain in the lymphoid organs for a long time. Of these

a small percentage is released daily together with "old" lymphocytes to such an extent that the first-day cells represent about one half per cent of the cells in the blood stream. If the amount of lymphoid tissue in man is about 1 kg, as assumed by HELLMAN (1935), then the tissue may contain about 2×10^{12} lymphocytes while the blood may contain about 10^{10} lymphocytes. If the lymphocytes in the blood stream are renewed twice in 24 hours, the average time a lymphocyte spends in the organs may be estimated as $\frac{2 \times 10^{12}}{2 \times 10^{10}} = 100$ days. Thus, the long life span of the main part of the lymphocytes is presumably explained even if a circulation of the lymphocytes, like that assumed by SJÖVALL (1936), occurs to only a minor extent, as found by YOFFEY and DRINKER (1939). The hypothesis that many of the new-formed lymphocytes spend a rather long time in the lymphoid organs before they enter the blood stream is in agreement with experiments on patients with chronic lymphatic leukemia (CHRISTENSEN and OTTESEN 1954).

This explanation is not inconsistent with the rapid turnover of DNA phosphorus in rat lymphoid organs as found in experiments of a few hours duration by ANDREASEN and OTTESEN (1945). The results of a few preliminary long-term experiments on old rats favour the assumption that a fraction of the DNA phosphorus in lymph glands and spleen is renewed rapidly, while another fraction is renewed slowly and thus may be located in long-living lymphocytes stored in the glands. All the DNA phosphorus in the thymus is renewed rapidly (ANDREASEN and OTTESEN 1952).

FICHTELIUS (1951, 1953) found a biphasic appearance of ^{32}P-labelled lymphocytes in the blood of rabbits, sheep and goats. The specific activity curves for the lymphocyte DNA phosphorus had maxima at about 3 days and, later, at about 9 to 15 days. The time before the second maximum was shortened when the blood loss of the animal was increased above that due to the normal blood sampling. By repeated heavy bleeding, the second maximum could be abolished in rabbits. In the present experiments, the maximum at about 3 days corresponds to the first maximum found by FICHTELIUS. No second maximum was established. The purpose of the present experiments, however, was mainly to demonstrate the long life span of the major part of the lymphocytes. More elaborate experiments are required to provide

evidence on possible maxima during the later part of the experiments.

It is of interest to note that in patients B. and H. the maximum of the lymphocyte curve and that of the granulocyte curve are so widely apart in time that most of the short-living lymphocytes are allowed to enter the bone marrow and to differentiate there into granulocytes. During this process, the labelling of the DNA phosphorus should be retained. The $\varphi(t)$ curve of the granulocytes indicates only the fraction of the blood DNA phosphorus in the granulocytes which was formed during the first day of the experiment. The same $\varphi(t)$ values are obtained whether this fraction remained in the bone marrow all the time from its formation to its discharge, or whether the fraction was formed in the lymphoid organs and later transported to the bone marrow by means of lymphocytes, subsequently undergoing transfer into the blood in the form of granulocytes. Thus, the experiments reported in the present paper do not contradict the conclusions of FARR (1951) who transfused rabbit lymphocytes autogenously after labelling in vitro. Twelve hours after the transfusion, some of the cells which had gone into the marrow were found to be transformed into granulocytes.

The author wishes to express his sincere gratitude to Professor G. HEVESY for kind and stimulating interest and helpful suggestions throughout the work. I am deeply indebted to Professor N. BOHR and Professor P. BRANDT REHBERG for numerous facilities put at my disposal, to Professor H. H. USSING for valuable discussions, and to dr. med. B. CHR. CHRISTENSEN for kindly administering the radiophosphorus and securing the blood samples from the patients. Furthermore, I am grateful to dr. med. E. ANDREASEN for helpful advice.

Summary.

A method is described for the separation of granulocytes and lymphocytes from human blood.

The white cells are labelled during their formation by incorporation of radioactive phosphorus into their desoxyribonucleic acid. Evidence is presented for the assumption that the radioactive phosphorus incorporated in the desoxyribonucleic acid during the formation of the cells remains in the cells during their lifetime. From the specific activity data, it is possible to calculate the time the cells have spent in the organism, from the time of their la-

belling until they are sampled from the blood stream. This time is called the age of the cells.

The mean age of the granulocytes is found to be 8.7 and 9.4 days. The main part of the granulocytes enters the blood stream at an age of about 6 days. Less than 5 per cent of the granulocytes in the blood stream are less than 5 days old, and a negligible percentage is older than 3 weeks.

The lymphocytes form two groups, one younger than 10 days with a mean age of about 3 to 4 days, the other having a mean age of about 100 to 200 days. In one experiment, the short-living fraction represented 22 per cent, the long-living 78 per cent of the lymphocytes in the blood. In the other experiment, the short-living cells comprised about 11 per cent, the long-living 89 per cent of the total population in circulation.

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On the Mechanism of Intestinal Fat Absorption in the Cat.

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In a previous investigation (BERGSTRÖM, BORGSTRÖM, CARLSTEN and ROTTENBERG 1950) we studied the intestinal absorption of glycerides containing labelled stearic acid in different combinations on anaesthetized cats with a thoracic duct fistula. Rather large differences were then observed in the specific activities of the lymph phospholipids after feeding corn oil containing a small amount of free dissolved stearic-1-^{14}C acid, on the one hand, and when feeding the fatty acids obtained by hydrolysis of this mixture, on the other hand. These differences have not been found in experiments on unanaesthetized rats with the main intestinal lymphatic cannulated (BERGSTRÖM and BORGSTRÖM 1954). A possible explanation of this might be that the anaesthesia maintained during the cat experiment had interfered with the normal absorption mechanism. Only 3—12 per cent of the lipids fed were in the lymph, a fact also indicating that intestinal fat absorption was impaired. This question has now been studied in unanaesthetized cats in which the main intestinal lymphatics had been cannulated by an abdominal approach.

Experimental.

Preparation of the animals:

Cats weighing about 3 kg were used. Only domestic animals were chosen and for some days before the operation they repeatedly ex-

perienced the experimental conditions until they became sufficiently accustomed to them. Then food was withheld for 24 hours and they were operated upon in the following way:

Under ether anaesthesia the mesentery was exposed through an epigastric midline incision. On both sides of the superior mesenteric artery lie lymphatics. In this region all visible lymphatics were tied off as far away from the gut as possible. The largest lymph trunk was dissected free and a plastic cannula 10—15 cm in length was inserted about 5 mm into the peripheral part of the trunk. This cannula, treated with "Silicone", had an outer diameter of about 3 mm, but was stretched in moderate heat to provide a segment of about half this diameter. Where this thinner portion tended to broaden the cannula was cut. The tubing thus treated tapered to a point except at its extremity, which was slightly thickened. The tip could then easily be retained within the vessel by a ligature. To hold the cannula in the line of the lymphatic, a second ligature was placed about 5 mm central to the first. Through a small opening just caudal to the right costal margin the cannula was exteriorized.

To prevent the cannula from being pulled out of the lymphatic, the animal was enveloped in a jacket of calico provided with apertures for the limbs. In the right part of the jacket there was an opening for the cannula.

The experiments were performed on the second or third day after the operation. No food was given during the previous 24 hours. The cats remained during the whole experiment in a quiet, moderately warm room, where they had been trained to lie comfortably. The fatty material was given orally with the aid of a syringe. The exteriorized part of the cannula was connected through the opening of the jacket to a piece of plastic tubing, 20—25 cm in length, which carried the lymph to a beaker chilled with dry ice.

As a rule the colourless lymph became white $^1/_2$—1 hour after the administration of the fat and remained so for 10—15 hours. A person sat quietly in the vicinity of the cat during the whole experiment for the purpose of soothing the animal and correcting changes in its position that could interfere with the collection of the lymph. Physiological saline was given subcutaneously in quantities approximately equivalent to the amount of lymph collected.

In some animals two or three experiments could be performed, and in these cases at least 48 hours had to pass before a new experiment was commenced.

With the technique described a permanent lymph fistula has been observed to exist for more than one week and the animal remained in good condition. For the sake of accuracy the animals were not used more than three times, because it has been demonstrated that anastomoses are developed in cats about 10 days after tying off a large lymph trunk (LEE 1923, CARLSTEN and OLIN 1952).

Stearic acid-1-^{14}C was used in combination with corn oil as seen in table 1.

The techniques of extraction, separation and isolation of the fatty

Table 1.

Form of fat administered	Cat no.	Collection time in hours	Lymph volume (ml)	Total lymph fat (mg)	% of fed fat recovered in lymph	% of phospholipids in total lymph fat
A. Corn oil + free labelled stearic acid	VI[1]	12	63	1,296	52.1	6.8
	VII[1]	10	42	1,410	72.6	8.4
	IV[1]	15	74	1,352	51.7	6.8
	IV[2]	13	—	1,395	23.1	4.3
B. Corn oil transesterified with labelled stearic acid	IV[3]	12	48	986	31.4	10.6
C. Hydrolysed corn oil + free labelled stearic acid	VI[2]	12	72	1,272	39.2	11.1
	VII[2]	11	90	1,222	35.5	11.5
D. No fat administered (fasting cat)	VIII	17	60	360	—	23

Table 2.

Form of fat administered	Cat no.	Specific activities of lymph phospholipid fatty acids in per cent of total fatty acids of the fed mixture	Specific activities of lymph glyceride fatty acids in per cent of specific activity of the fed fatty acid mixture
A. Corn oil + free labelled stearic acid	VI[1]	63.7	82.2
	VII[1]	120.0	99.9
	IV[1]	94.2	80.7
	IV[2]	52.1	36.4
B. Corn oil transesterified with labelled stearic acid	IV[3]	67.2	78.8
C. Hydrolysed corn oil + free labelled stearic acid	VI[2]	64.7	72.0
	VII[2]	71.8	72.4

acids from the different lymph lipid fractions were essentially the same as those earlier used (1950).

Results and discussion.

The results are summarized in tables 1 and 2 and show that in these experiments much higher absorption percentage was

observed in the unanaesthetized cats than in the anaesthetized cats in the previous investigation (1950). In the anaesthetized cats the recovery of labelled fat in the lymph was only between 12.8 and 4.3 per cent. In this investigation the highest and lowest figures were 72.6 and 23.1 per cent respectively. It must be observed that these recoveries are calculated on the amount fed and not on the amount absorbed from the intestine as in most similar experiments on rats. The varying figures must be ascribed partly to the anatomical condition in the cat. Only the largest intestinal lymph duct was cannulated. Some smaller ones were tied off and some were presumably left by passing the fistula. The data indicate, however, that the results are approximating those obtained in unanaesthetized rats even if the experiment conditions probably placed more stress on the cats. The change of technique from a cannula in the thoracic duct to a cannula in the main intestinal lymphatic cannot be responsible for the difference found. In the cat the route of the intestinal lymph has been shown to be only via the thoracic duct (CARLSTEN and OLIN 1952).

In this investigation the relative specific activities of the lymph phospholipid fatty acids are also somewhat higher in series A than in the other series but the differences are much smaller and irregular than in experiments on unanaesthetized rats. As it has been found (BORGSTRÖM 1952) that a preferential absorption of free fatty acids occurs during absorption in the rat this might also be the explanation for the differences of the lymph phospholipid fatty acids in the different series reported here. Probably one of the reasons why the preferential absorption of free fatty acid was even more pronounced in the anaesthetized cats in which the normal absorption mechanism was obviously disturbed, was the low intestinal motility with a subnormal action of the pancreatic lipase leading to a subnormal dilution of the preferentially absorbed free labelled fatty acid by unlabelled acid set free from the corn oil.

The results presented confirm that there is no difference in the route of absorption of labelled stearic acid when fed as glyceride or when dissolved in free fatty acids. The distribution of the labelled acid in the lymph glycerides and phospholipids also show that an extensive hydrolysis and randomization has taken place. These results indicate, in contradistinction to MELLANBY's (1927) opinion, that lipases play an important rôle in the triglyceride

absorption of the normal cat as in other mammals that have been investigated.

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Summary.

The intestinal absorption of ^{14}C-labelled fat has been studied in the unanaesthetized cat with a main intestinal duct fistula. The results are in accordance with those obtained on rats in corresponding experiments. Their results amplify those previously obtained on anaesthetized cats with the thoracic duct cannulated. The differences are most probably explained by the effect of the anaesthesia.

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